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The soluble guanylyl cyclase activator YC-1 increases intracellular cGMP and cAMP via independent mechanisms in INS-1E cells*

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Running title: sGC activator YC-1 directly increases cAMP

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Abbreviations: YC-1, 5-[1-(phenylmethyl)-1H-indazol-3-yl]-2-furanmethanol; NO, nitric oxide; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; NS-2028, 8-bromo-4H-2,5-dioxa-3,9b-diaza-cyclopenta[a]naphthalen-1-one; KH7, 2-(1H-benzimidazol-2-ylthio)-2-[(5-bromo-2-hydroxyphenyl)methylene]hydrazide, propanoic acid; 2’5’ ddAdo, 2’5’ dideoxyadenosine; sAC, soluble adenylyl cyclase; sGC, soluble guanylyl cyclase; tmAC, transmembrane adenylyl cyclase; IBMX, 3-isobutyl-1-methylxanthine; cAMP, 3'-5'-cyclic adenosine monophosphate; cGMP, 3'-5'-cyclic guanosine monophosphate; ERK, extracellular signal-regulated kinases.

ABSTRACT

In addition to increasing cGMP, the soluble guanylyl cyclase (sGC) activator 3-(5'-hydroxymethyl-2'-furyl)-1-benzyl indazole (YC-1) can elevate intracellular cAMP levels. This response was assumed to be as a result of cGMP dependent inhibition of a cAMP phosphodiesterases (PDE); however, in this study we show that YC-1 induced cAMP production in the rat pancreatic beta cell line, INS-1E cells occurs independent of its function as sGC activator and independent of its ability to inhibit PDEs. This YC-1-induced cAMP increase is dependent upon soluble adenylyl cyclase (sAC) and not on transmembrane adenylyl cyclase (tmAC) activity. We previously showed sAC-generated cAMP can lead to ERK activation, and YC-1 stimulated cAMP production also stimulates ERK. While YC-1 has been used as a tool for investigating sGC and cGMP-mediated pathways, this study reveals cGMP-independent pharmacological actions of this compound.

Introduction

The derivative of benzylindazole, 3-(5'-hydroxymethyl-2'-furyl)-1-benzyl indazole (YC-1), was the first of a class of compounds identified to directly activate soluble guanylyl cyclase (sGC). Work on purified sGC (Mulsch et al., 1997) revealed that YC-1 stimulates sGC in a heme-dependent and nitric oxide (NO)-independent manner (Ko et al., 1994; Wu et al., 1995; Friebe et al., 1996). YC-1 can sensitize the enzyme for NO activation *in vitro* (Friebe et al., 1996), in human platelets (Teng et al., 1997) and in smooth muscle (Galle et al., 1999), and it has been shown to enhance ERK and CREB phosphorylation in amygdala and hippocampus through a NO-cGMP-PKG pathway (Chien et al., 2003; Chien et al., 2008). Thus, YC-1 has been a valuable reagent for exploring sGC/cGMP-dependent signaling pathways in biological systems.

YC-1 has other reported effects as well. It inhibited the activity of cyclic nucleotide catabolizing phosphodiesterases (PDE) in aortic extracts, specifically isoforms 1-5 (Galle et al., 1999). This effect would augment the elevation of cGMP due to stimulation of sGC. Other reported activities of YC-1, which were found to be independent of its effects on sGC activity, included stimulating NO production in endothelial cells (Wohlfart et al., 1999), protecting optic nerves (Garthwaite et al., 2002), inhibiting voltage dependent K^+ channels in rabbit coronary arterial smooth muscle cells (Park et al., 2010), inhibiting respiratory burst and degranulation in human neutrophils (Hwang et al., 2003), and inhibiting proliferation of mesangial cells through p38 MAPK activation (Chiang et al., 2005).

YC-1 has also been reported to elevate levels of the distinct second messenger, cAMP. In platelets (Ko et al., 1994) and human neutrophils (Hwang et al., 2003), YC-1 increases cAMP (and PKA activity). Such effects were postulated to be mediated via inhibition of a putative cGMP-inhibited cAMP PDE (Ko et al., 1994).

During the course of our studies exploring the role of cAMP in beta cells of the pancreas, we found that YC-1 also elevated cAMP, independent of any effect on cGMP, in the beta cell-like insulinoma cell line INS-1E. Beta cells produce cAMP when exposed to high concentrations of glucose (Charles et al., 1973; Grill and Cerasi, 1973; Rutter, 2001; Tian et al., 2011), and this response can be studied in INS-1E cells (Ramos et al., 2008). We previously demonstrated that the bicarbonate, calcium and ATP-regulated soluble adenylyl cyclase (sAC) is at least partially responsible for the glucose-induced cAMP generation in INS-1E cells, and that glucose-induced ERK phosphorylation is exclusively dependent upon sAC-generated cAMP (Ramos et al., 2008). We now demonstrate that YC-1 elevates cAMP accumulation in INS-1E cells independent of its effects on sGC or cAMP catabolizing PDEs, and dependent upon sAC. Thus, although it has long been appreciated that cAMP levels can be affected during YC-1 treatment, we now demonstrate that such effects are cGMP independent, and reveal a unique, unknown direct target of YC-1 actions.

Methods

Reagents

INS-1E cells were a gift from Claus Wollheim, University Medical Center, Geneva, Switzerland. RPMI media, L-glutamine and HEPES were acquired from CellGrow (Manassas, VA). The β-mercaptoethanol, dimethylsulfoxide, sodium pyruvate and glucose were obtained from Sigma-Aldrich (St. Louis, MO) and Fetal Bovine Serum (FBS) from Gemini Bio-Products (West Sacramento, CA). Phospho-ERK and total ERK rabbit polyclonal antibodies were purchased from Cell Signaling Technology (Danvers, MA), Horseradish peroxidase-linked anti-mouse and anti-rabbit antibodies, and SuperSignal West Pico chemiluminescent substrate were obtained from Thermo Fisher Scientific (Rockford, IL). The anti-sAC monoclonal antibody, R21 was developed in our laboratory, and is directed against amino acids 203-216 of human sAC.

The following reagents were used in this study: YC-1, ODQ, NS2028 and BAY 41-8543 (Cayman Chemical Co, Ann Arbor, MI). The S-nitroso-N-acetylpenicillamine (SNAP) and 2’5’ dideoxyadenosine were obtained from EMD Chemicals (Gibbstown, NJ) and 3-isobutyl-1-methylxanthine (IBMX) was from Sigma (St. Louis, MO). The sAC specific inhibitor, KH7 was synthesized by ChemDiv, Inc (San Diego, CA) and by the Abby and Howard P. Milstein Synthetic Chemistry Core Facility of Weill Cornell Medical College.

Cell Culture

INS-1E cells (passage 150 – 175) were cultured as previously described (Asfari et al., 1992). The cells were passaged every three days and cultured under 5% CO_2 in RPMI media supplemented with 10% heat-inactivated fetal bovine serum (FBS), 10 mM

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HEPES, 1 mM sodium pyruvate, and 50 μM β-mercaptoethanol.

Adenylyl cyclase assays and cAMP determinations

In vitro adenylyl cyclase activity was measured on purified recombinant rat sAC_t protein as previously described (Litvin et al., 2003) (Buck et al., 1999; Chen et al., 2000). For *in vivo* cAMP accumulation assays, $2.5x10^5$ INS-1E cells were plated in each well of a 24-well plate. Two days later, cells were incubated in 2.5 mM glucose Krebs-Ringer buffer (pH 7.5) supplemented with 2 mM sodium bicarbonate, 10 mM HEPES, and 0.1% bovine serum albumin (BSA) for 1 – 2 h before the start of the assay. At time zero, media was replaced with Krebs-Ringer buffer containing 2.5 mM glucose or 16 mM glucose in the presence of 500 μM IBMX and the different inhibitors or vehicle controls as specified in figure legends. We found no difference in accumulated cAMP levels whether IBMX was pre-incubated for 10 minutes or added simultaneously with glucose or the various drugs (data not shown). Cells were incubated for the indicated time at 37 °C, followed by aspiration of media and the cells were lysed with 200 μL of 0.1 M hydrochloric acid (HCl) per well. Intracellular cAMP content was determined using Correlate-EIA cAMP Direct Assay (Assay Designs, Inc).

For assays of *in vitro* transcription/translation products, rat full-length (sAC_{fl}) and truncated (sAC_t) soluble adenylyl cyclase cDNAs were expressed (Buck et al., 1999). *In vitro* transcription/translation was performed using TNT Quick-Coupled Transcription/Translation System (Promega Corp., Madison, WI) according to manufacturer's instructions. Synthesis of sAC_{fl} and sAC_t proteins were confirmed by

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Western blot with sAC monoclonal antibody, R21. For measuring cAMP production, 15 μL of *in vitro* transcription/translation products were assayed in 100 μL of 200 mM Tris (pH 7.5), 5 mM ATP, 20 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM NaH_2CO_3, 0.5 mM IBMX in the presence of vehicle control or KH7 or YC-1 as specified in figure legends. Cyclase reactions were incubated at 30 ºC for 20 min and stopped with 100 μL of 0.2 M HCl. Cyclic AMP produced was measured by using the Correlate-EIA Direct cAMP Enzyme Immunoassay Kit (Assay Designs, Inc.)

Guanylyl cyclase assays and cGMP determinations

$2.5x10^5$ INS-1E cells were plated in each well of a 24-well plate. The assay was performed the same way as the adenylyl cyclase assay described above. Intracellular cGMP content was determined using Correlate-EIA cGMP Direct assay (Assay Designs, Inc).

Phosphodiesterase assays and cAMP determination

INS-1E cells were grown in 10 cm dishes to 80% confluence. Cells were lysed in cold lysis buffer (150 mM NaCl, 1 mM EDTA, 1 mM DTT, 50 mM Tris pH 8.0) in the presence of 10 μg/mL aprotinin, 10 μg/mL leupeptin, 5 mM benzamidine and 1 mM phenylmethanesulfonylfluoride (PMSF). To measure PDE activity, 1/20 of total lysate was used per reaction in the presence of 25 pmol cAMP. Lysates were incubated in 200 mM Tris (pH 7.5), 20 mM $MgCl_2$, 3 mM DTT and in the presence or absence of IBMX, YC-1 and other PDE inhibitors as indicated in figure legends for 30 min at 37 °C. To stop the assay, 0.2 M HCl was added, and intracellular cAMP content was determined

using Correlate-EIA Direct Assay (Assay Designs, Inc). Assays were performed in triplicate.

Western Blot

Equal numbers of cells were plated on 6-well dishes. Cyclic AMP accumulation assays were performed in the presence of inhibitors or activators as specified in the figure legends. After 15 min of incubation, 100 μL of Laemmli sample buffer was added directly to the cells in the wells. 10 – 15 μL of sample was resolved on an SDS-PAGE, transferred to a PVDF membrane, and probed with specific antibodies (i.e., anti-phospho-ERK, or anti-ERK antibodies).

RESULTS

YC-1 increases cAMP production in INS-1E cells independent of sGC

In INS-1E cells, incubation in high (16 mM) glucose elevates intracellular cAMP (Ramos et al., 2008). YC-1 increased cAMP accumulation in INS-1E cells in both low (2.5 mM) and high (16 mM) glucose (Figure 1A). YC-1 stimulation was dose dependent and seemed to be additive with the effect of high glucose. At 100 μM, which is the level of YC-1 required for maximal activation of purified sGC (Friebe and Koesling, 1998), cAMP accumulation was stimulated approximately two-fold compared to the cAMP level in the absence of drug under both low and high glucose conditions. YC-1 induced cAMP generation was specific to this drug; a structurally unrelated activator of sGC, BAY 41-8543 (Stasch et al., 2002), had no effect on cAMP production in INS-1E cells (Figure 1B).

The absence of a cAMP increase by BAY 41-8543 suggested that the YC-1-induced increase in cAMP production would be independent of sGC. We tested this directly by including two pharmacological inhibitors of sGC, ODQ or NS2028 (Garthwaite et al., 1995; Olesen et al., 1998). After confirming that neither sGC inhibitor affected cAMP levels in INS-1E cells under low or high glucose (Figure 2), we found that they also had no effect on the YC-1-induced cAMP rise (Figure 3A). As expected, both YC-1 or BAY 41-8543 stimulated cGMP accumulation in INS-1E cells, and both ODQ and NS 2028 inhibited cGMP production induced by either (Figure 3B). Thus, YC-1's effect on cAMP appears to be independent of its stimulation of sGC.

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YC-1 induces cAMP accumulation independent of its ability to inhibit PDE activity

cAMP accumulates as a consequence of an imbalance between second messenger generation by adenylyl cyclases (ACs) and its degradation by catabolizing phosphodiesterases (PDEs). YC-1 has reported effects on PDE activity (Ko et al., 1994; Galle et al., 1999; Hwang et al., 2008), so we explored whether its ability to elevate intracellular cAMP was mediated by inhibition of a cAMP catabolizing PDE. To directly test whether YC-1 affected PDE activity in INS-1E cells, we measured cAMP PDE activity in INS-1E lysates in the presence of YC-1 alone, or in concert with the broad specificity PDE inhibitor IBMX and the more selective dipyridamole, which potently inhibits the only known IBMX-insensitive cAMP-catabolizing PDE, PDE8 (Fisher et al., 1998; Soderling et al., 1998). YC-1 alone slightly decreased the cAMP-PDE activity in INS-1E lysates; its effect was approximately equal to the effect of the PDE8 inhibitor dipyridamole (Figure 4A). However, the effects of YC-1 was completely abrogated in the presence of 0.5 mM IBMX, suggesting that its effect on PDE activity in INS-1E cells is mediated through inhibition of an IBMX sensitive PDE isoform. The cellular cAMP accumulation assays described above (Figures 1-3) were performed in the presence of IBMX, suggesting that YC-1's effects were not due to PDE inhibition. We retested this by repeating the cellular cAMP accumulation experiments in the presence of both dipyrimidole and IBMX. Dipyridamole (in the presence of IBMX) had a slight, but not statistically significant, effect on the level of cAMP accumulation in INS-1E cells, and YC-1 still induced a cAMP rise when both IBMX and dipyridamole were present (Figure

4B). Therefore, while YC-1 may exhibit some PDE inhibiting ability, its ability to diminish cAMP catabolizing activity cannot be solely responsible for its elevation of cAMP in whole cells.

YC-1-induced cAMP production requires sAC activity.

Since YC-1 did not appear to be increasing cAMP solely by inhibiting a PDE activity, we hypothesized that it should be stimulating AC activity. In mammalian cells in general, and in INS-1E cells in particular (Ramos et al., 2008), there are two classes of adenylyl cyclase; a family of G protein regulated transmembrane adenylyl cyclases (tmACs) and bicarbonate-, calcium- and ATP-regulated soluble adenylyl cyclase (sAC). We previously established the use of small molecule inhibitors selective for each class to distinguish which AC was responsible for a specific cAMP signaling cascade (Stessin et al., 2006; Wu et al., 2006; Ramos et al., 2008). KH7 is a small molecule which specifically inhibits sAC (Hess et al., 2005), and the P site ligand, 2'5' dideoxyadenosine (2'5'ddAdo), is selective for tmACs when used at $\leq$ 50 μM (Johnson et al., 1997; Gille et al., 2004). Using these inhibitors, we demonstrated that in INS-1E cells, incretin-stimulated cAMP is generated by tmACs while sAC is responsible for the cAMP induced by elevated glucose (Ramos et al., 2008). Similar to what we previously observed (Ramos et al., 2008), 2'5'ddAdo, but not KH7, diminished the "basal" cAMP accumulation in low glucose (cAMP amounts in low glucose in the absence of any inhibitor = 12.976 ± 0.442 pmol/well, in the presence of KH7 = 13.286 ± 0.815 pmol/well, and in the presence of 2'5'ddAdo = 9.755 ± 1.384 pmol/well). In contrast, the YC-1 induced cAMP was

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inhibited by KH7 and unaffected by 2'5'ddAdo (Figure 5). Thus, the cAMP increase observed in the presence of YC-1 is dependent upon sAC, and not tmACs.

We tested the effect of YC-1 on the activities of the two sAC isoforms, sAC_t and sAC_{fl}, which have been characterized *in vitro* (Buck et al., 1999; Jaiswal and Conti, 2003; Chaloupka et al., 2006). The truncated and highly active sAC_t isoform can be heterologously expressed and purified (Chen et al., 2000; Litvin et al., 2003), while sAC_{fl} has thus far only ever been assayed in whole cell lysates from transfected cells (Chen et al., 2000; Jaiswal and Conti, 2003; Chaloupka et al., 2006) or in immunoprecipitates (Jaiswal and Conti, 2003). We now demonstrate that active sAC_{fl} can be produced and assayed by *in vitro* transcription/translation systems. In our hands, YC-1 had no effect on basal sAC_t or sAC_{fl} adenylyl cyclase activities, nor did it potentiate calcium and bicarbonate stimulation of sAC_t (Figure 6).

YC-1 induced cAMP stimulates the MAP kinase cascade

In beta cells, elevated glucose activates the MAP kinase cascade, and in our previous work, we demonstrated that glucose-induced, sAC-generated cAMP is required for phosphorylation of the MAP kinase, ERK (Ramos et al., 2008). We now show that the YC-1-induced, sAC-dependent cAMP rise also stimulates the MAP kinase cascade. YC-1 treatment increases ERK phosphorylation, although not to the same extent as high glucose (Figure 7). And this YC-1 induced increase is blocked by the sAC inhibitor KH7, but not by the sGC inhibitors ODQ or NS2028. Thus, YC-1 stimulates cAMP

accumulation in a manner consistent with elevated glucose; it is dependent upon sAC and results in MAP kinase cascade activation.

DISCUSSION

In this study, we demonstrate that YC-1 has effects in INS-1E cells, which are independent of its known functions as an sGC activator. We have yet to determine its specific target, but YC-1 induces cAMP accumulation independent of its effects on cGMP generation. The source of the cAMP induced by YC-1 is sAC, and the YC-1 induction of second messenger occurs when all known phosphodiesterases are inhibited. These observations reveal that, at least in INS-1E cells, YC-1 raises intracellular cAMP via a unique, unknown direct target.

Consistent with its demonstrated ability to stimulate sGC activity, YC-1 activates cGMP accumulation in INS-1E cells. However, using YC-1 to probe intracellular signaling must include confirmation by sGC inhibition (i.e., ODQ or NS 2028) and by independent (and more selective) sGC activation (i.e., by BAY 41-8543). In contrast, YC-1 remains a useful reagent for studying sGC activation *in vitro*. A great deal of effort has been invested in understanding how it binds to the enzyme, yet where YC-1 binds to sGC remains a matter of debate (Friebe et al., 1999; Derbyshire et al., 2009). Some mutational studies indicated that YC-1 interacts with the catalytic domain of sGC (Russwurm et al., 2002); however, photoaffinity labeling and other mutational studies suggested that it binds within the alpha1 subunit linker region between the Heme-NO and PAS domains (Stasch et al., 2001; Koglin and Behrends, 2003). Such a mechanism may be consistent with Resonance Raman studies, which revealed that YC-1 binding caused a conformational change, which resulted in heme adjustment (Ibrahim et al., 2010).

In 1999, Galle *et al*. showed that YC-1 inhibited the activities of the cGMP specific PDE5 as well as the activities of the non-selective (i.e., cAMP and cGMP catabolizing) PDE1, PDE2, and PDE3 (Galle et al., 1999). Each of these cAMP-catabolizing isoforms are sensitive to inhibition by IBMX (Soderling and Beavo, 2000; Boswell-Smith et al., 2006). There are also cAMP-catabolizing PDEs which are IBMX-insensitive (or whose sensitivity to IBMX is unknown). PDE8 is an IBMX-insensitive, cAMP-selective PDE (Soderling et al., 1998), while PDE10 and PDE11 are non-selective PDEs whose IBMX sensitivity remains unclear. Fortunately, all three isoforms (along with cGMP specific PDE5 and PDE6) can be inhibited by dipyridamole (Hetman et al., 2000; Ghosh et al., 2009), and in INS-1E extracts, dipyridamole's small but significant ability to inhibit PDE activity seemed to be additive with IBMX. Hwang and coworkers showed that YC-1 increased cAMP accumulation in human neutrophils, and because their effect was not seen in the presence of IBMX, they concluded YC-1 elevated cAMP via inhibition of an IBMX-sensitive PDE (Hwang et al., 2003). Similarly, we observed a small, but significant, decrease in cAMP-PDE activity in extracts from INS-1E cells in the presence of YC-1. Consistent with YC-1 inhibiting an IBMX-sensitive PDE, the effect of YC-1 seemed to be additive with dipyridamole and absent in the presence of IBMX. In any event, in the presence of both IBMX and dipyridamole, YC-1 had no effect on PDE activity *in vitro*, yet it was still able to stimulate intracellular cAMP accumulation in INS-1E cells. These results suggest YC-1 affects cAMP accumulation independent of its ability to inhibit PDEs. However, it remains possible that YC-1's inhibitory effects on

PDEs are greater or more efficient *in vivo* than *in vitro*, or that it effects a PDE activity not reflected in the *in vitro* PDE assay.

The YC-1 induced cAMP elevation is inhibited by the sAC-specific inhibitor KH7, while selective inhibition of tmAC activity had no effect. Therefore, YC-1 induction of cAMP requires sAC activity to generate the second messenger. Yet, we were unable to demonstrate that sAC was the target of YC-1; the two biochemically characterized sAC isoforms were inert when tested *in vitro*. Other sAC isoforms derived from an internal promoter are predicted to exist (Geng et al., 2005; Farrell et al., 2008). These are likely to be distinctly regulated, and they may be the target of YC-1.

It also remains possible that YC-1 increases sAC activity by modulating the intracellular concentration of one of its regulators. sAC catalytic activity is directly regulated by bicarbonate (Chen et al., 2000) and calcium (Jaiswal and Conti, 2003; Litvin et al., 2003), and its affinity for substrate ATP (approximately 1 mM) suggests it will be sensitive to intracellular fluctuations of ATP (Litvin et al., 2003). All three modulators may contribute to the glucose-induced stimulation of sAC activity in INS-1E cells (Ramos et al., 2008). Glucose metabolism leads to increased intracellular ATP and CO_2/bicarbonate, and because INS-1E are beta-cell like, the glucose-dependent rise in intracellular ATP closes the ATP-regulated potassium channel (K_{ATP}) which depolarizes the cell, opening a voltage gated calcium channel (VDCC) and elevating intracellular calcium (Ashcroft and Rorsman, 1989; Rutter et al., 1993). We previously demonstrated that the glucose-dependent stimulation of sAC in INS1-E cells is dependent upon the VDCC-mediated

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calcium rise, and the sAC response could be mimicked by potassium depolarization (Ramos et al., 2008). YC-1 can inhibit other types of potassium channels (Park et al., 2010), suggesting that YC-1 may activate sAC subsequent to depolarization induced calcium entry. However, YC-1 was able to induce approximately the same fold stimulation of cAMP accumulation in both low and high glucose (Figure 1), implying that glucose and YC-1 increase cAMP via distinct mechanisms. Finally, there are additional modes of regulation of sAC_{fl} yet to be understood (Chaloupka et al., 2006)., and therefore, it remains possible that YC-1 elevates intracellular cAMP via modulation of one of these known, or unknown, sAC activators.

Future studies examining whether YC-1 also elevates cAMP in other contexts, and whether any observed cAMP rise is sAC-dependent and sGC and PDE-independent, may help shed light on its precise mechanism of action. In conclusion, we identified an effect of YC-1 which is entirely independent of its sGC activating properties. Due to such unwanted consequences, caution should be taken in the interpretation of results when using this compound, especially since there are known pathways where cAMP and cGMP modulate the same physiological effect, either oppositely or in concert.

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Authorship Contributions:

Participated in research design: Ramos-Espiritu, L.S., Levin, L.R., and Buck, J.

Conducted the experiments: Ramos-Espiritu, L.S., and Hess, K.

Contributed new reagents or analytical tools: Ramos-Espiritu, L.S., Hess, K., Levin, L.R., and Buck, J.

Performed data analysis: Ramos-Espiritu, L.S., Hess, K., Levin, L.R., and Buck, J.

Wrote or contributed to the writing of the paper: Ramos-Espiritu, L.S., Levin, L.R., and Buck, J.

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Figure Legends

Figure 1. *YC-1, but not Bay 41-8543, potentiates cAMP production in both low and high glucose conditions in INS-1E cells.* Two days before the assay, $2.5x10^5$ INS-1E cells were plated in each well of a 24-well plate. (A) Total cellular cAMP was measured in INS-1E cells in Krebs Ringer buffer after incubation for 15 min with 2.5 mM glucose or 16 mM glucose in the presence of 0.5 mM IBMX, with either vehicle control (0), or with YC-1 at corresponding concentrations. Values represent means ± S.E.M (n= 4) of total cAMP content per well. (B) Cyclic AMP was measured in INS-1E cells after incubation for 15 min in 2.5 mM glucose in the presence of 0.5 mM IBMX, with either vehicle control or BAY 41-8543. Values represent means ± S.E.M (n= 3). ANOVA statistical analyses were performed with the Bonferroni post-test. ***, $P < 0.0001$, **, $P < 0.001$, *, $P < 0.01$.

Figure 2. *Soluble guanylyl cyclase inhibitors have no effect on glucose-induced cAMP production in INS-1E cells.* Two days before the assay, $2.5x10^5$ INS-1E cells were plated in each well of a 24-well plate. Total cellular cAMP was measured in INS-1E cells in Krebs Ringer buffer with 2.5 mM glucose or 16 mM glucose in the presence of 0.5 mM IBMX, in 2.5 mM glucose or 16 mM glucose with the corresponding amounts of sGC inhibitors; ODQ (A), NS2028 (B), Values represent means ± S.E.M (n= 3) of total cAMP content per well.

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Figure 3. *Soluble guanylyl cyclase inhibitors do not inhibit YC-1-induced cAMP production in INS-1E cells.* Two days before the assay, $2.5x10^5$ INS-1E cells were plated in each well of a 24-well plate. (A) Cyclic AMP was measured in INS-1E cells after incubation for 15 min in 2.5 mM glucose in the presence of 0.5 mM IBMX, with either vehicle control or 100 μM YC-1 combined with sGC inhibitors, 100 μM ODQ and 100 μM NS2028 and (B) Cyclic GMP was measured in INS-1E cells after incubation for 15 min in 2.5 mM glucose in the presence of 1.0 mM IBMX and 100 μM SNAP with either vehicle control or 100 μM YC-1 or 100 μM BAY 41-8543, combined with sGC inhibitors, 100 μM ODQ and 100 μM NS2028. Values represent means ± S.E.M (n= 4). ANOVA statistical analyses were performed with the Bonferroni post-test. **, $P < 0.001$.

Figure 4. *YC-1-induced cAMP accumulation is not due to PDE inhibition.* (A) Sensitivity of cAMP-hydrolyzing activity in INS-1E lysate to PDE inhibitors, 0.5 mM IBMX and 30 μM dipyridamole, and to 100 μM YC-1. Values represent means ± S.E.M (n= 4). (B) Two days before the assay, $2.5.0x10^5$ INS-1E cells were plated in each well of a 24-well plate. Cyclic AMP was measured in INS-1E cells after incubation for 15 min in 2.5 mM glucose in the presence of 0.5 mM IBMX, with either vehicle control or 30 μM dipyridamole and/or with 100 μM YC-1. Values represent means ± S.E.M (n= 4). ANOVA statistical analyses were performed with the Newman-Keuls post-test. **, $P < 0.001$, NS, $P>0.05$.

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Figure 5. *YC-1-induced cAMP production is inhibited by a sAC-specific inhibitor.* Cyclic AMP was measured in INS-1E cells after incubation for 15 min in 2.5 mM glucose in the presence of 0.5 mM IBMX, with either vehicle control (basal), 100 μM YC-1 and YC-1 combined either with 30 μM KH7 or 50 μM 2'5'ddAdo. The data are presented as fold stimulation of basal cAMP accumulation. Values represent means ± S.E.M (n= 4).

Figure 6. *Activities of soluble adenylyl cyclase isoforms, sAC*t *and sAC*$_{fl}$ are unaffected by *YC-1*. (A) Adenylyl cyclase assays were performed using recombinant rat sAC$_t$ at the indicated concentrations of YC-1 in the presence of 50 mM Tris-HCl pH 7.5 and 10 mM $MgCl_2$, 10 mM ATP (basal) (circle) or 10 mM $MgCl_2$, 10 mM ATP, 10 mM $NaHCO_3$, and 0.5 mM $CaCl_2$ (sub-optimal) (square) for 30 minutes at room temperature. Values represent averages of triplicate determinations. (B) Synthesized sAC$_{fl}$ and sAC$_t$ from *in vitro* transcription/translation were assayed for adenylyl cyclase activity. Cyclase assays were performed in 100 μL of total reaction volume using 15 uL of *in vitro* transcription/translation products combined with reaction mix composed of: 200 mM Tris (pH 7.5), 5 mM ATP, 20 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM NaH_2CO_3, 0.5 mM IBMX and with vehicle control or 30 μM KH7 or 100 μM YC-1. MM is adenylyl cyclase reaction conditions alone (no *in vitro* transcription/translation products). Values represent means ± S.E.M (n=3). (C) Western blot using sAC monoclonal antibody, R21 to verify the synthesis of sAC$_t$ (lane 1- 50 KDa) and sAC$_{fl}$ (lane 2- 187 KDa) protein in *in vitro* transcription/translation system.

Figure 7. *Glucose and YC-1 lead to ERK (p42/44) activation in a sAC-dependent manner.* Western blots using anti-phosphoERK (pERK) or total ERK (ERK) of INS-1E cells incubated in Krebs Ringer buffer with 16 mM glucose (HG) or 2.5 mM glucose (LG) for 30 minutes in the presence or absence of 100 μM YC-1 or combination of YC-1 with 30 μM KH7, 100 μM ODQ and 100 μM NS2028. Shown are representative experiments repeated multiple times (n=3).

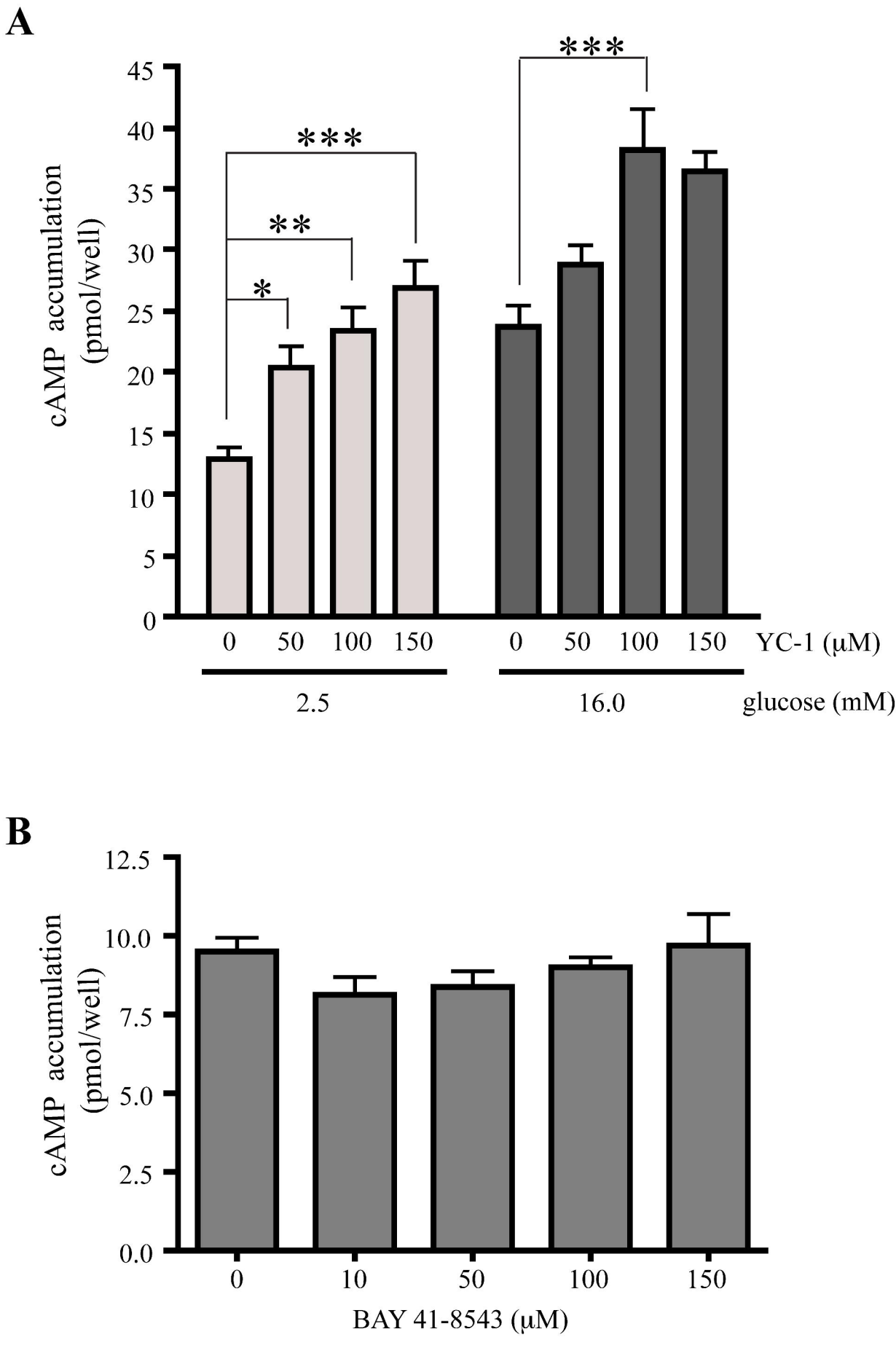
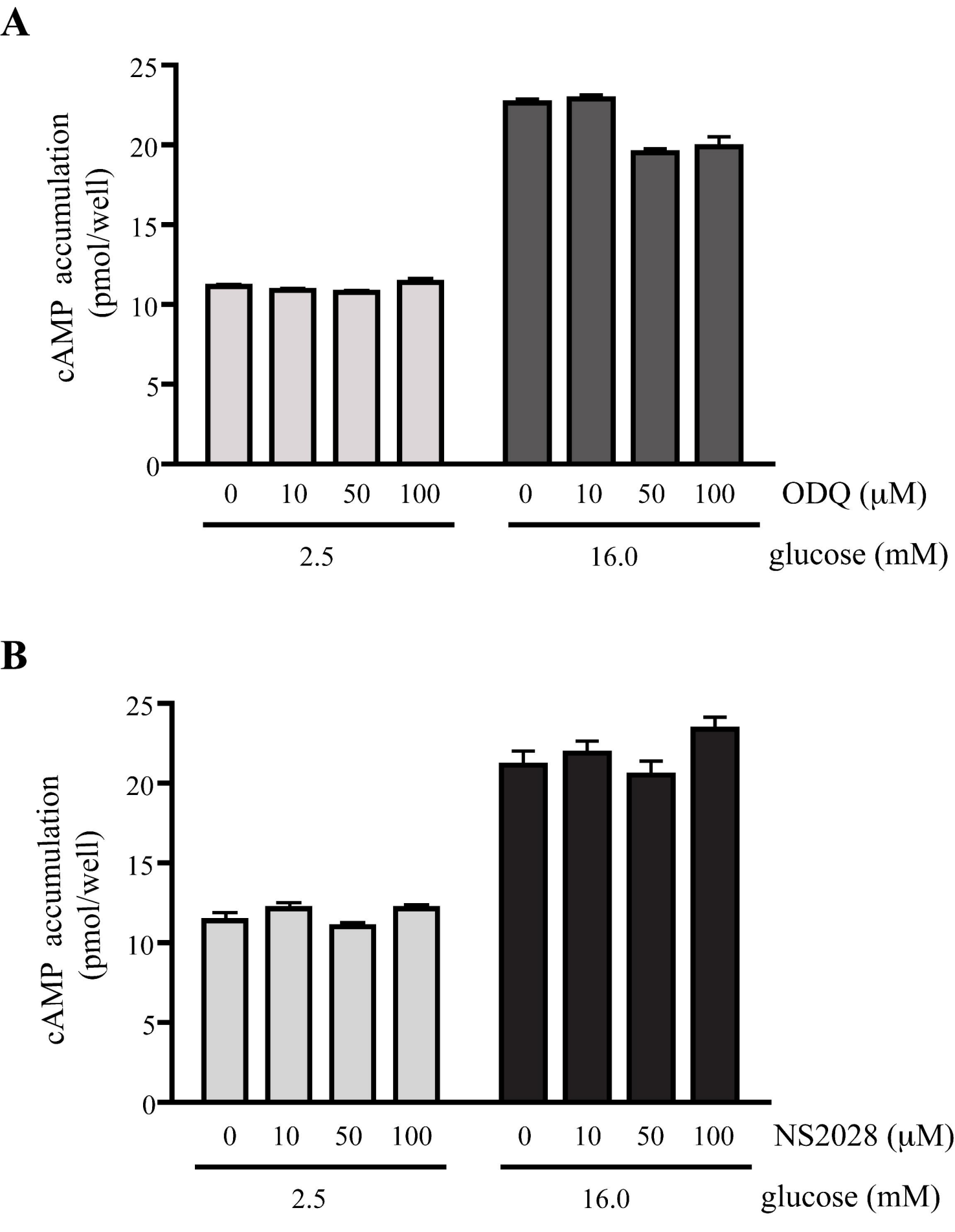

A
cAMP accumulation (pmol/well)
45
40
35
30
25
20
15
10
5
0
***
**
*
***
0
50
100
150
0
50
100
150
YC-1 (μM)
2.5
16.0
glucose (mM)
B
cAMP accumulation (pmol/well)
12.5
10.0
7.5
5.0
2.5
0.0
0
10
50
100
150
BAY 41-8543 (μM)

Figure 1


A
cAMP accumulation (pmol/well)
25
20
15
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ODQ (μM)
2.5
16.0
glucose (mM)
B
cAMP accumulation (pmol/well)
25
20
15
10
5
0
0 10 50 100 0 10 50 100
NS2028 (μM)
2.5
16.0
glucose (mM)

Figure 2

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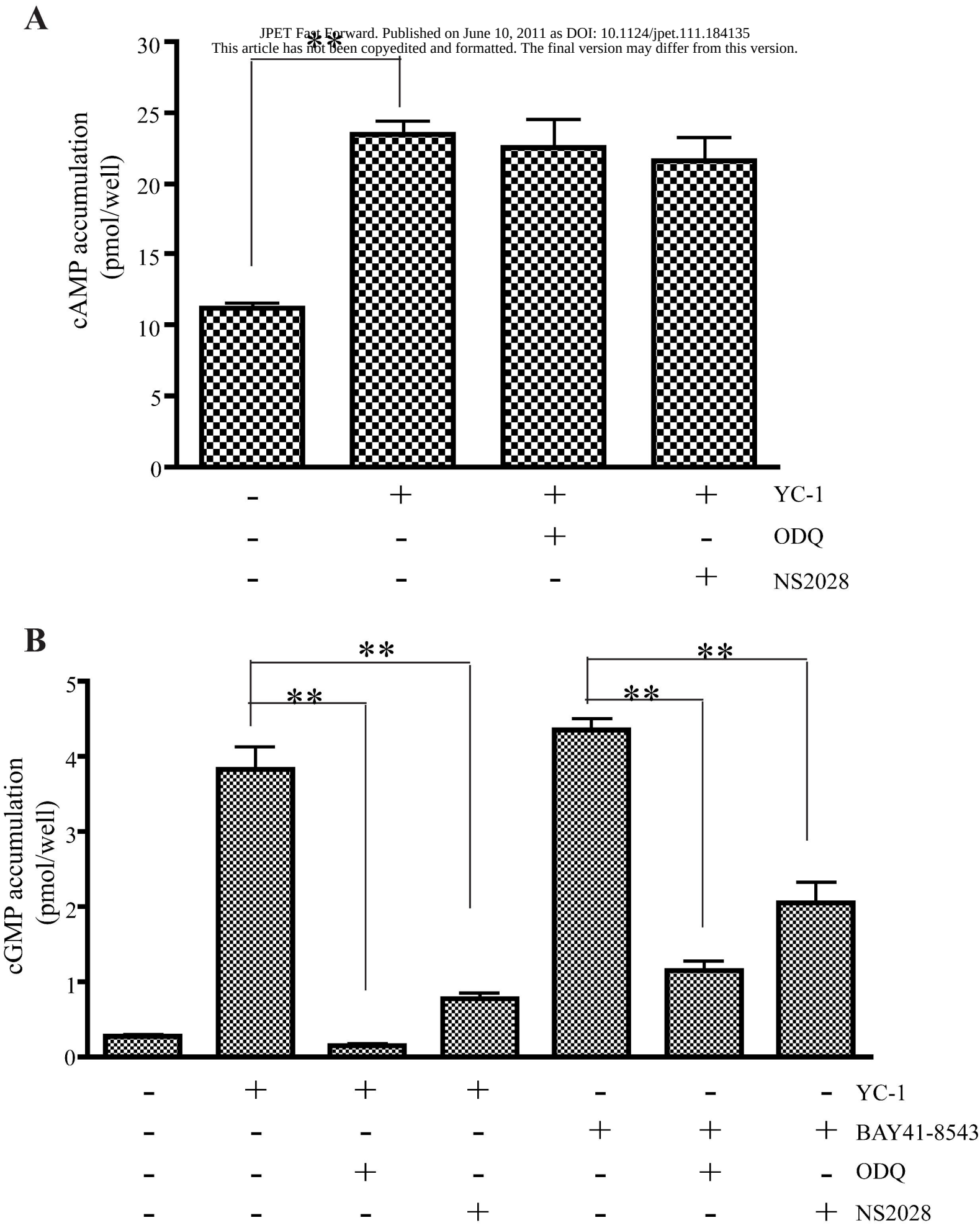

Figure 3

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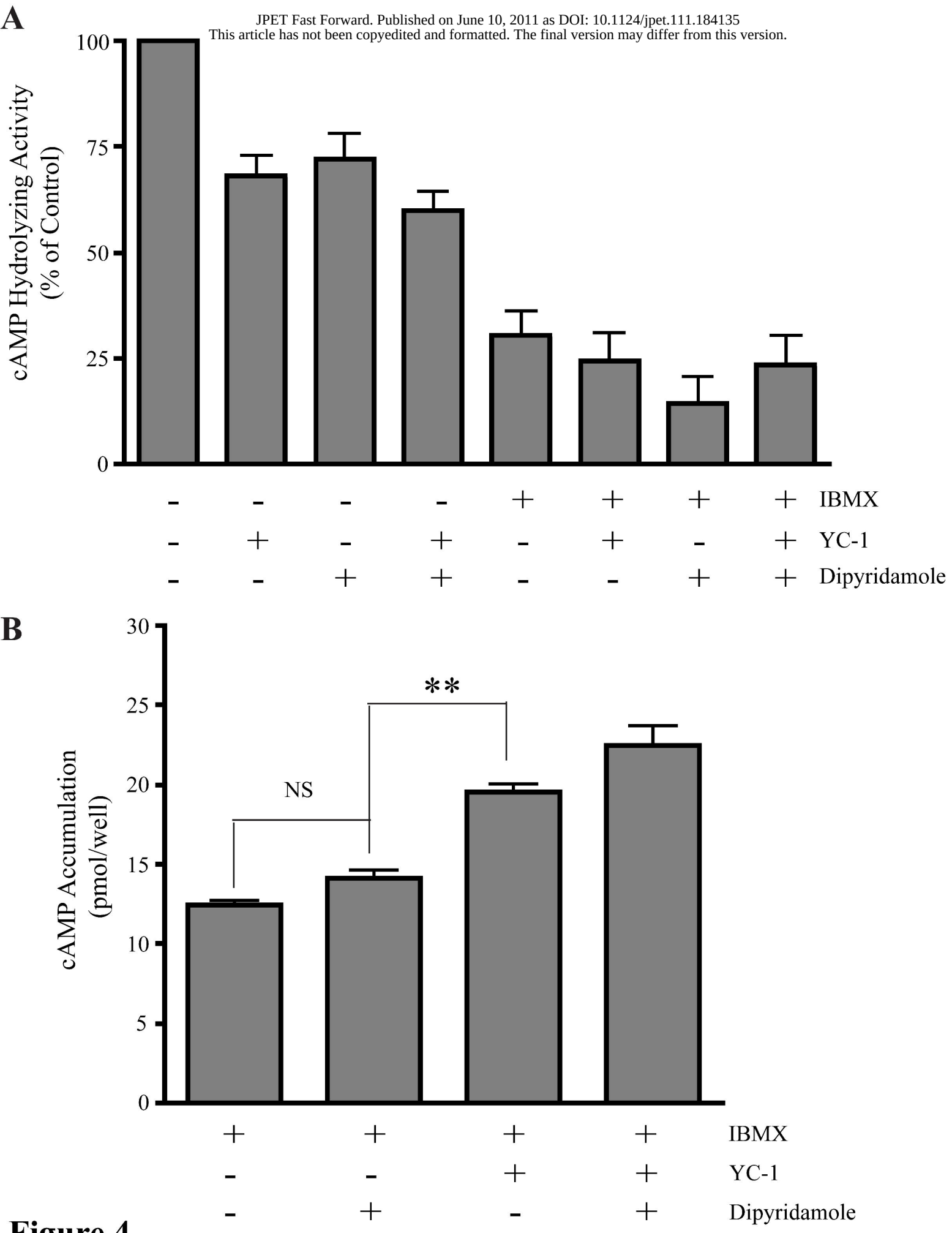

Figure 4

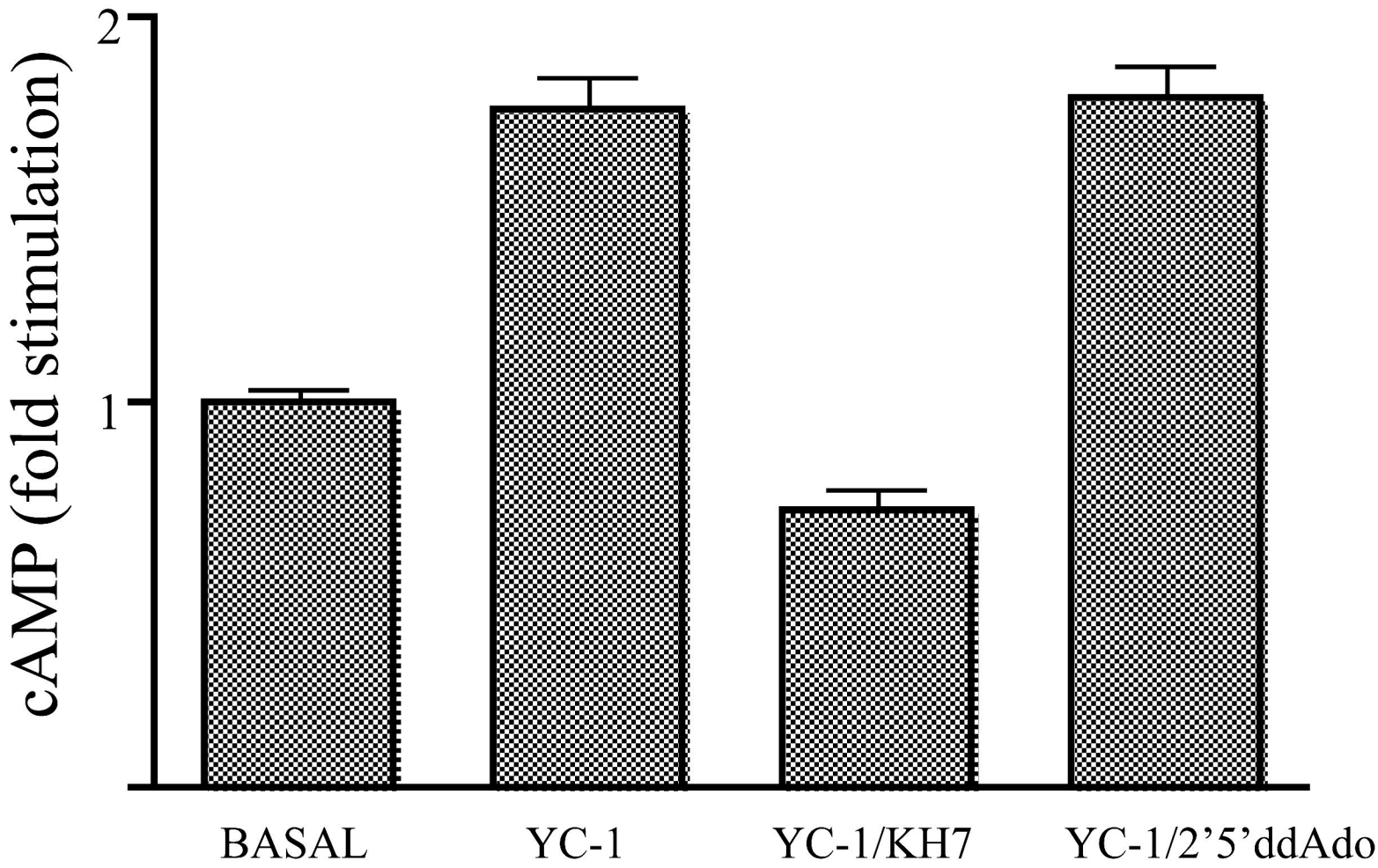

Figure 5

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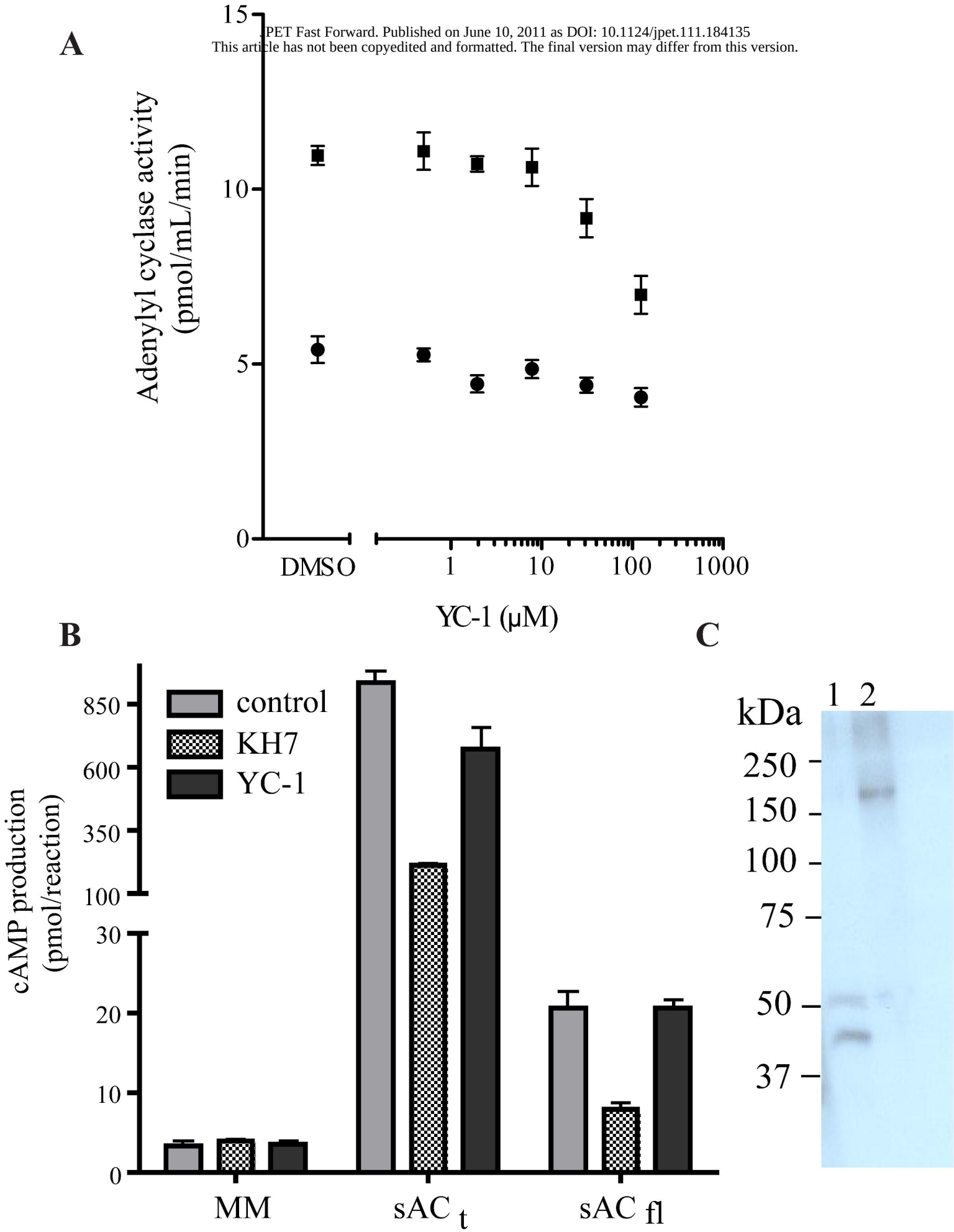

Figure 6

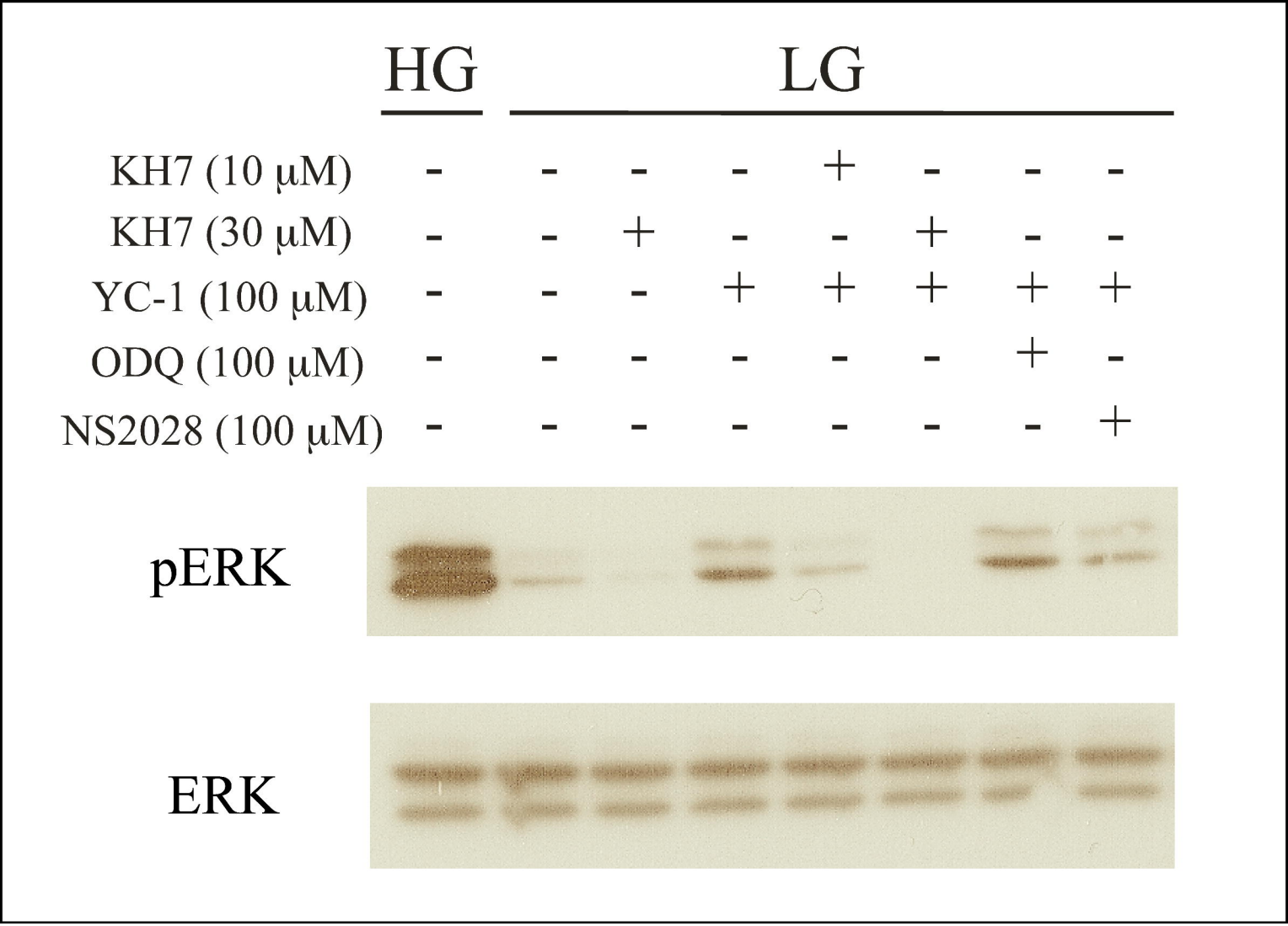
HG
LG
KH7 (10 μM) - - - - + - - -
KH7 (30 μM) - - + - - + - -
YC-1 (100 μM) - - - + + + + +
ODQ (100 μM) - - - - - - + -
NS2028 (100 μM) - - - - - - - +
pERK
ERK

Figure 7