Supporting Information

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SI Materials and Methods

Reagents used in this study include Fluo-4/Fura-2 (Invitrogen), thapsigargin (Calbiochem), collagenase (Worthington), palmitoleic acid ethyl ester (POAEE) (Cayman Chemical), and GSK-7975A from GSK (2,6-difluoro-*N*-(1-(4-hydroxy-2-(trifluoromethyl)benzyl)-1*H*-pyrazol-3-yl)benzamide), compound 36 from patent WO2010/122089 A1 (1). All other chemicals were purchased from Sigma. Pancreatic acinar cells were isolated from mice as described previously (2). The extracellular solution contained: 140 mM NaCl, 4.7 mM KCl, 10 mM Hepes (KOH), 1 mM $MgCl_2$, 10 mM glucose, pH 7.2, and $CaCl_2$ (0–10 mM as described in the text). Changes in the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) were monitored by Fluo-4 or Fura-2 fluorescence measurements and necrosis assessed by propidium iodide (PI) uptake (3). We assessed protease activation with the generic fluorescent substrate bis-L-aspartic acid amide rhodamine 110 (D2-R110) and trypsinogen activation with the trypsin fluorescent substrate Rhodamine 110, bis-(CBZ-L-isoleucyl-L-prolyl-L-arginine amide), dihydrochloride (BZiPAR) (4, 5). Fluorescence signals were plotted as F/F_0 (F_0 is the initial level of fluorescence). The polynomial curve fit method was used to estimate IC_{50}. Statistical significance and *P* values were calculated using *t* test or one-way ANOVA. Data presented as mean ± SEM. In Na^+-free solution, Na^+ was replaced by N-methyl-D-glucamine ($NMDG^+$). During standard patch clamp whole-cell recordings, the pipette solution contained 120 mM KCl, 1 mM $MgCl_2$, 2 mM MgATP, 15 mM Hepes, 10 mM BAPTA and 2 mM $CaCl_2$. Patch pipettes were pulled from borosilicate glass capillaries (Harvard Apparatus). The pipettes had a resistance of 3–5 megaohms when filled with an intracellular solution (containing 120 mM KCl). Whole cell currents were sampled at 10 KHz using an EPS-8 amplifier and Pulse software (HEKA) or MultiClamp700B amplifier and pClamp 10 software (Molecular Devices). In the standard protocol the membrane voltage was clamped at −50 mV. For investigations of current–voltage relationships voltage ramps were applied from −100 to + 40 mV (the slope was 400 mV/s). Changes in $[Ca^{2+}]$ in the intracellular stores were assessed by fluorescence measurements in cells loaded with Fluo-5N AM (Invitrogen) simultaneously with measurements of whole cell currents.

The protocol for primary hepatocyte isolation was as described in ref. 6. All animal work was carried out in accordance with the UK Animals (Scientific Procedures) Act (1986) and approved by the Ethical Review Committee of Cardiff University. Briefly, the liver was perfused with buffer I without Ca^{2+}: 140 mM NaCl; 4.7 mM KCl; 10 mM Hepes; 10 mM D-glucose; 100 μM EGTA; (pH 7.4); the rate of perfusion was 5 mL/min at 37 °C. Then the liver was perfused with buffer I in the presence of 1.3 mM $CaCl_2$ and collagenase I (Sigma) for 10 min at 37 °C. Dissociated hepatocytes were centrifuged at 50 × *g* for 1 min and transferred into buffer I containing 1 mM $MgCl_2$ and 1.3 mM $CaCl_2$, pH 7.4.

AR42J cells (ECACC, 93100618) were maintained in RPMI 1640 as described previously (3, 7).

For intracellular calcium imaging isolated hepatocytes were loaded with fluo-4 in AM form at room temperature for 45 min.

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6. Li W-C, Ralphs KL, Tosh D (2010) Isolation and culture of adult mouse hepatocytes. *Methods Mol Biol* 633:185–196.
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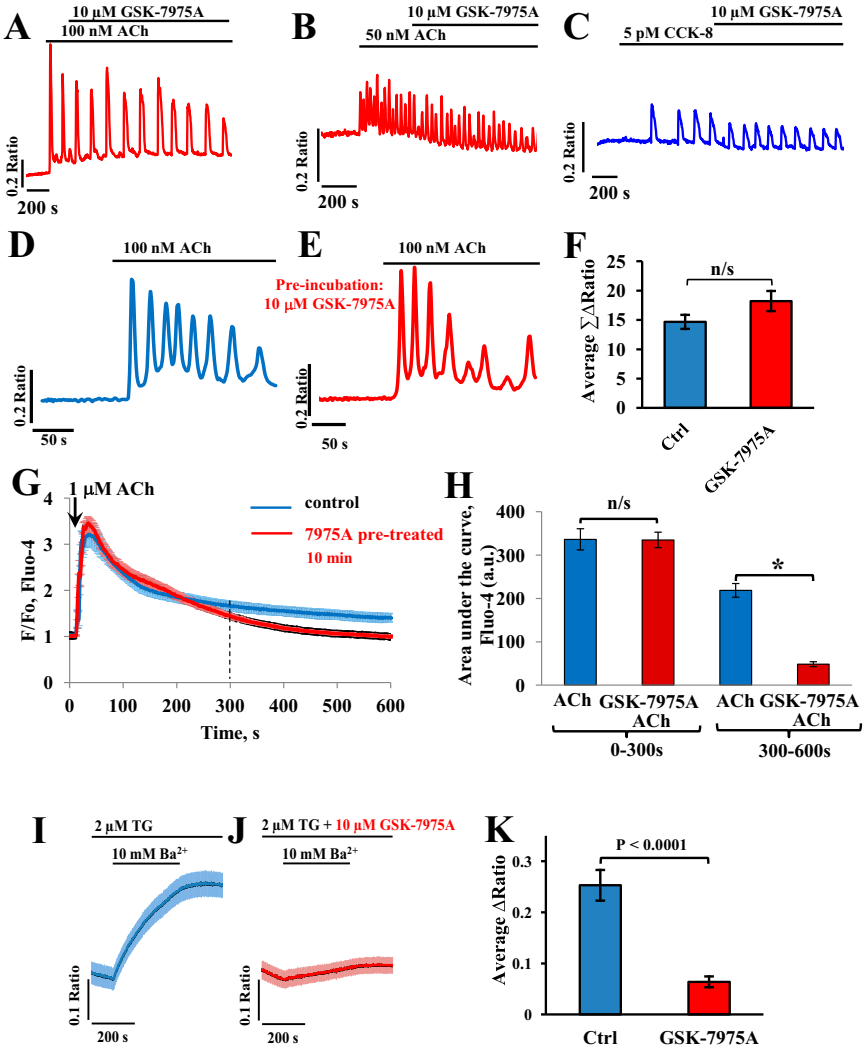

Fig. S1. GSK-7975A does not affect $[Ca^{2+}]_i$ spike generation induced by low concentrations of ACh or CCK. (*A–C*) Acute application of 10 μM GSK-7975A does not interrupt Ca^{2+} oscillations induced by low (quasiphysiological) concentrations of ACh [100 nM (*A*) and 50 nM (*B*)] or a low physiological concentration of CCK [5 pM (*C*)]. (*D* and *E*) Preincubation with 10 μM GSK-7975A does not reduce Ca^{2+} oscillations induced by a low concentration (100 nM) of ACh (*E*, $n = 29$) compared with control (*D*, $n = 23$). (*F*) Comparison of the areas under $[Ca^{2+}]_i$ changes induced by 100 nM ACh in the presence or absence of 10 μM GSK-7975A. Data represent mean values ± SEM. $P > 0.1$ (n/s, nonsignificant difference). Experiments were performed in standard buffer containing 1 mM $CaCl_2$. (*G*) GSK-7975A inhibits the sustained phase of the $[Ca^{2+}]_i$ elevation induced by a high concentration of ACh. Average traces of $[Ca^{2+}]_i$ changes in response to 1 μM ACh in control cells (blue trace, $n = 14$) and in cells preincubated with 10 μM GSK-7975A for 10 min (red trace, $n = 21$). Bars represent ±SEM. (*H*) Quantitative analysis of experiments in *G*. Averaging $[Ca^{2+}]_i$ elevations induced by ACh recorded during the first 300 s (10–310 s) shows similar values (area under the curve, 336.3 ± 28.5 and 335.1 ± 18.4 au, n/s $P = 0.97$). However, very different values were obtained during the last 300 s (300–600 s) in cells pretreated with GSK-7975A (10 μM) (red bars) and control cells (blue bars) (48.3 ± 5.1 and 218.7 ± 16.3 au, $*P < 10^{-6}$). (*I–K*) Average traces of $[Ba^{2+}]_i$ rise (together with SEM) due to store-operated Ba^{2+} influx in the presence (*J*, $n = 38$) or absence (*I*, $n = 36$) of 10 μM GSK-7975A. (*K*) Comparison of the amplitudes of the increases in $[Ba^{2+}]_i$ in response to admission of Ba^{2+} to the external solution (from *I* and *J*) in the presence and absence of 10 μM GSK-7975A, $P < 0.0001$.

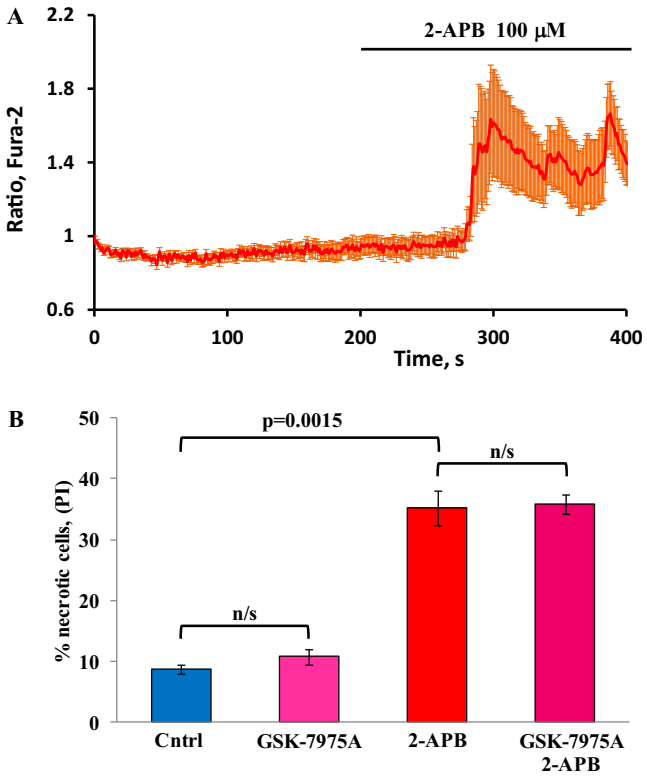

Fig. S2. Blocker of CRAC channels 2-APB induces rise in $[Ca^{2+}]_i$ and necrosis in pancreatic acinar cells, which cannot be inhibited by GSK-7975A. (*A*) Averaged traces (together with error bars) of rise in $[Ca^{2+}]_i$ induced by 100 μM 2-APB ($n = 14$). (*B*) Preincubation of pancreatic acinar cells with 100 μM 2-APB induces substantial necrosis (35 ± 2%, $P < 0.0015$, $n = 4$ series, with >80 cells in each). GSK-7975A does not induce significant necrosis by itself compared with control ($P > 0.2$) and does not protect against the necrosis induced by 2-APB ($P > 0.8$).

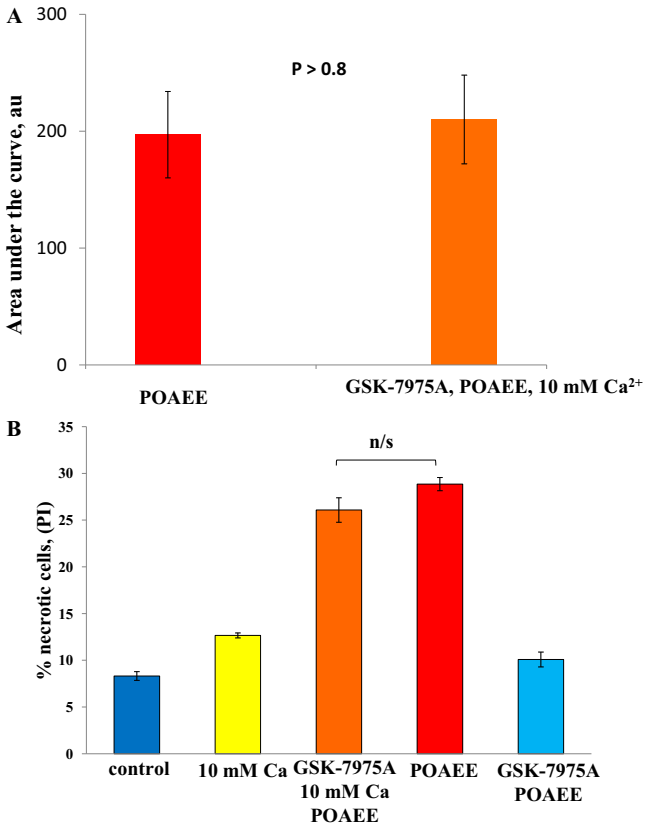

Fig. S3. Elevating the external Ca^{2+} concentration from 1 mM to 10 mM counteracts the protective effect of GSK-7975A against the POAEE-induced $[Ca^{2+}]_i$ rise and necrosis. (*A*) Comparison of the "areas under the curve" of the POAEE (100 μM)-induced $[Ca^{2+}]_i$ responses with the normal 1 mM external $[Ca^{2+}]$ ($n = 11$, typical example shown in Fig. 4*A*) and the responses induced by 100 μM POAEE in the presence of 10 mM external Ca^{2+} and GSK-7975A (10 μM) (cells pretreated with GSK-7975A for 10 min, $n = 26$). No significant difference was found ($P > 0.8$). (*B*) POAEE (100 μM)-induced necrosis (28.9 + 0.7% necrotic cells) was dramatically reduced in cells treated with 10 μM GSK-7975A for 10 min (10.1 ± 0.8%, $P < 0.003$, compared with POAEE treatment alone). However, if after treatment with GSK-7975A (10 μM, 10 min) POAEE (100 μM) was applied in the presence of 10 mM $CaCl_2$ in the external medium, the level of necrosis was not significantly different ($P > 0.14$) from POAEE-induced necrosis at an external Ca^{2+} concentration of 1 mM and without GSK-7975A (26 ± 1.3%). In a control series of experiments (no POAEE treatment, 1 mM $CaCl_2$), the level of necrosis was 8.3 ± 0.5%. The level of necrosis in the presence of 10 mM $CaCl_2$ increased to 12.7 ± 0.3% ($P < 0.0001$, compared with control). $n = 3$ for all series of experiments with number of tested cells in each group >350.

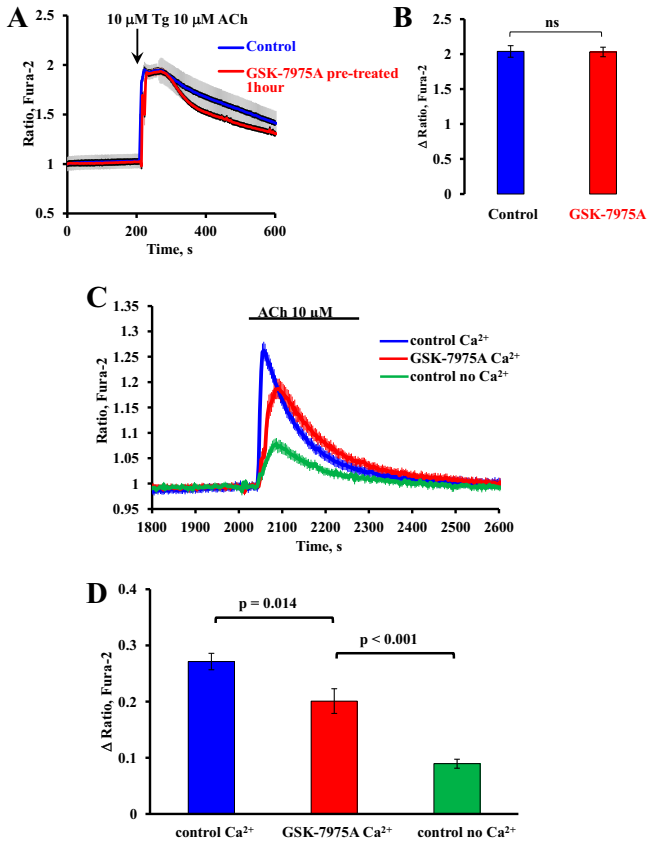

Fig. S4. GSK-7975A does not significantly affect the amount of Ca^{2+} that can be released from the ER in resting pancreatic acinar cells and only has a relatively minor effect on Ca^{2+} store reloading. (*A*) Cells were treated with 10 μM GSK-7975A for 1 h (red averaged trace (together with SEM), $n = 10$) and compared with control [blue averaged trace (together with SEM), $n = 14$], then external Ca^{2+} was removed (nominally Ca^{2+}-free solution) and cells were stimulated with 10 μM ACh together with 10 μM thapsigargin for maximal response. (*B*) Comparison of the maximal amplitudes of the rises in $[Ca^{2+}]_i$ shown in *A*. Control ($n = 14$) and GSK-7975A ($n = 10$) were not significantly different ($P > 0.05$). (*C*) Cells were stimulated with a high concentration of ACh (10 μM) for 10 min and then, after a long period of recovery, were stimulated for a second time with the same ACh concentration. The traces show the averaged second responses to ACh (together with SEM) in experiments carried out under three different conditions: standard external Ca^{2+} concentration (1 mM) without any blocker (blue trace, $n = 18$), in the presence of 10 μM GSK-7975A and 1 mM external Ca^{2+} (red trace, $n = 12$) and nominally Ca^{2+}-free external solution (green trace, $n = 12$). (*D*) Comparison of the amplitudes of the second responses to ACh in the traces shown in *C*. Averaged amplitude of the responses in control 1 mM external Ca^{2+} was 0.271 ± 0.015, $n = 18$. Averaged amplitude of the responses in the presence of GSK-7975A was slightly, but significantly lower (0.20 ± 0.022, $n = 12$, $P = 0.014$) than in control. The averaged amplitude of the response in the absence of external Ca^{2+} was substantially lower (0.089 ± 0.008, $n = 12$, $P < 0.001$) than in the presence of GSK-7975A and Ca^{2+}.

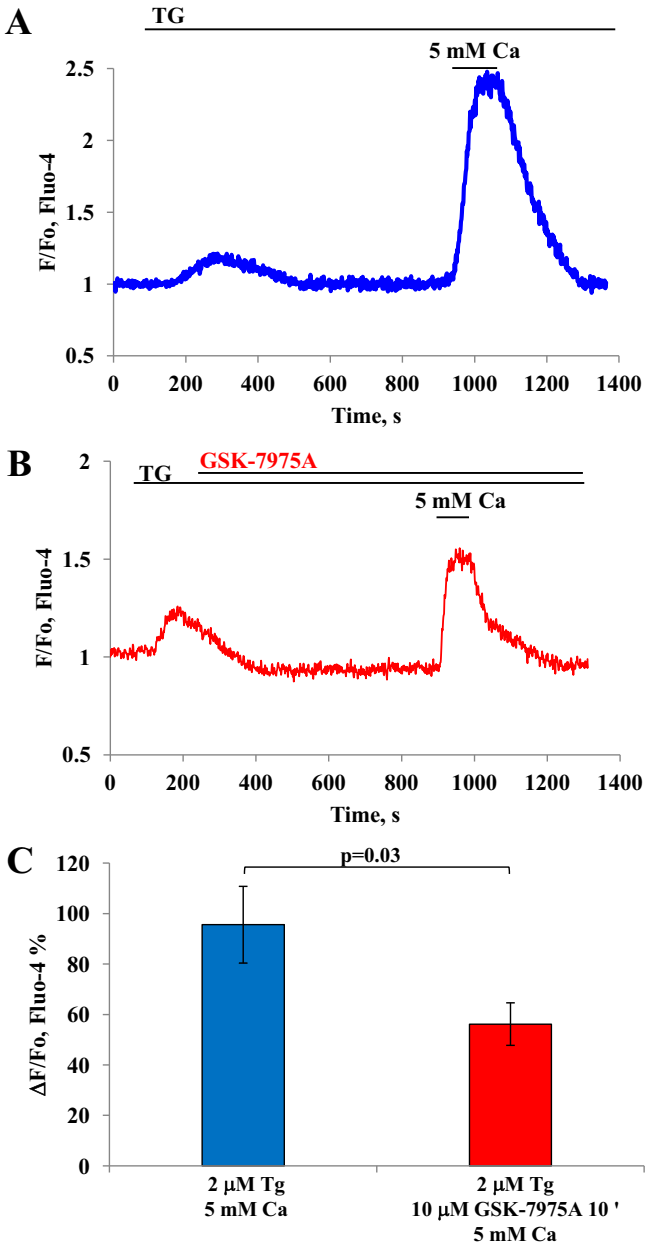

Fig. S5. Standard store-operated Ca^{2+} entry protocol (as in Fig. 1), but this time carried out in freshly isolated hepatocytes. GSK-7975A only has a relatively small inhibitory effect on store-operated Ca^{2+} entry. (*A*) Control representative trace of $[Ca^{2+}]_i$ changes evoked by thapsigargin (2 μM) in the absence of external Ca^{2+} and then following introduction of the external solution containing 5 mM Ca^{2+}. (*B*) Same protocol as in *A*, but this time in presence of 10 μM GSK-7975A. Store-operated Ca^{2+} entry has been reduced, but only to a limited degree. (*C*) Comparison of the responses induced by Ca^{2+} entry in control (95.6 ± 15 au, $n = 10$) and in the presence of 10 μM GSK-7975A (56.2 ± 8.4 au, $n = 11$). GSK-7975A has caused a relatively minor reduction in the amplitude of the Ca^{2+} entry response ($P < 0.03$).

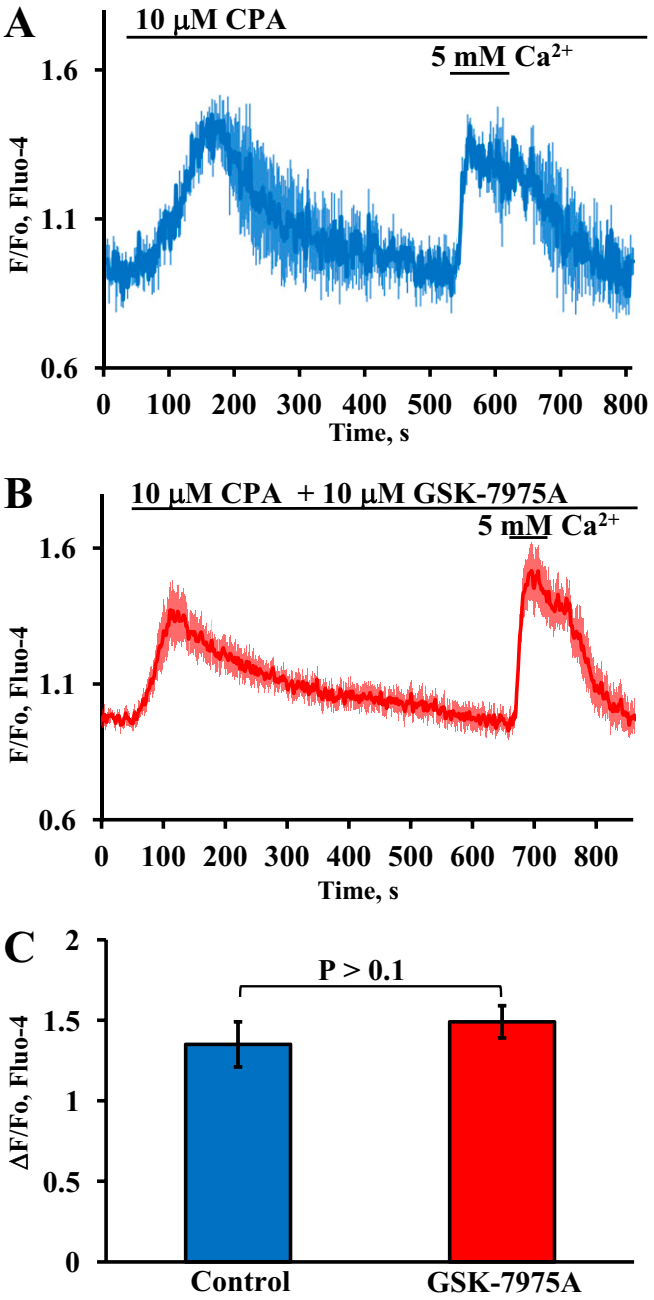

Fig. S6. GSK-7975A does not affect store-operated Ca^{2+} entry in rat pancreatic tumor cell line (AR42J). (*A*) Control averaged traces (together with error bars) of changes in $[Ca^{2+}]_i$ induced in AR42J cells first by exposure to 10 μM cyclopiazonic acid (CPA) in the absence of external Ca^{2+} and thereafter by introducing an external solution containing 5 mM Ca^{2+} ($n = 3$). (*B*) Same protocol as in *A*, but this time in the presence of 10 μM GSK-7975A ($n = 7$). The rise in $[Ca^{2+}]_i$ following admission of the external solution containing 5 mM Ca^{2+} is very similar to that observed in the absence of GSK-7975A. (*C*) Comparison of the amplitudes of the rise in $[Ca^{2+}]_i$ induced by store-operated Ca^{2+} entry in control (*A*) and in the presence of 10 μM GSK-7975A (*B*). There is no significant difference ($P > 0.1$).