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G Protein Coupled Receptor Signaled Apoptosis Is Associated with Activation of a Cation Insensitive Acidic Endonuclease and Intracellular Acidification

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Apoptosis associated oligonucleosomal fragmentation of DNA can result from the activation of endonucleases that exhibit different pH optima and are either sensitive or insensitive to divalent cations. DNA fragmentation due to activation of cation sensitive endonucleases occurs in the absence of a change in intracellular pH whereas intracellular acidification is a feature of apoptosis characterized by activation of cation insensitive acidic endonuclease. We have reported earlier that somatostatin (SST) induced DNA fragmentataion and apoptosis is signaled in a receptor subtype selective manner uniquely via human somatostatin receptor subtype 3 (hSSTR3). In the present study we investigated the pH dependence and cation sensitivity of endonuclease induced in hSSTR3 expressing CHO-K1 cells by the SST agonist octreotide (OCT) and its effect on intracellular pH. We show that OCT induced apoptosis is associated with selective stimulation of a divalent cation insensitive acidic endonuclease. The intracellular pH of of cells undergoing OCT induced apoptosis was 0.9 pH units lower than that of control cells. The effect of OCT on endonuclease and pH was inhibited by orthovanadate as well as by pretreatment with pertussis toxin, suggesting that hSSTR3 initiated cytotoxic signaling is protein tyrosine phosphatase mediated and is G protein dependent. These findings suggest that intracellular acidification and activation of acidic endonuclease mediate wild type p53 associated apoptosis signaled by hormones acting via G protein coupled receptors.

Apoptosis constitutes a specific form of cell death characterized by cell shrinking, chromatin condensation and intranucleosomal digestion of genomic DNA (1). In apoptotic cells DNA fragmentation results from the action of DNA digesting enzymes (endonucleases) whose activity is modulated by molecular regulators of apoptosis including p53 and Bax (2). Two distinct sets of constitutive endonucleases which exhbit distinct pH optima under *in vitro* conditions have been implicated in this process. These include several Ca^{2+} and/or Mg^{2+} dependent endonucleases including DNAse I, Nuc18 and endonuclease I which possess an alkaline pH optimum, DNAse γ which has a neutral pH optimum, and an acidic endonuclease that is active in the pH range of 6-7.5 (3–7). In addition, a Ca^{2+}/Mg^{2+} insensitive endonulcease (DNAse II) which has a pH optimum of 5 has also been shown to induce DNA degradation (8,9). Cation sensitive endonuclease mediated DNA fragmentation leads to cell death in the absence of a change in intracellular pH. By contrast, in several cell types in which DNA fragmentation is caused by the activation of a pH dependent endonuclease, apoptosis is associated with intracellular acidification (7,9–11).

Somatostatin (SST[1]), a G protein coupled receptor agonist, exerts cytotoxic effect triggering apoptosis as has been shown in tumor cells (12). Although SST acts via a family of five distinct human (h) SST receptor (SSTR) subtypes, cytotoxic signaling is transduced uniquely through hSSTR subtype 3 (13). We have shown that hSSTR3 signaled apoptosis in CHO-K1 cells is characterized by the appearance of oligonucleosomal DNA and is associated with the induction of the tumor suppressor protein wild type (wt) p53 and Bax (13). In order to determine which of the endonuclease(s) is/are activated during hSSTR3 mediated apoptosis and whether this is associated with intracellular acidification we investigated the effect of treatment with the somatostatin (SST) analog octreotide (OCT) on the endonuclease activity and intracellular pH in CHO-K1 cells stably transfected with hSSTR3. We report here that hSSTR3 signaled apoptosis in CHO-K1 cells is

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Abbreviations used: SST, somatostatin; SSTR, somatostatin receptor; OCT, octapeptide somatostatin analog octreotide; and PTx, pertussis toxin.

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due to selective activation of a pH dependent, cation insensitive endonuclease which occurs concomitantly with intracellular acidification.

MATERIALS AND METHODS

Materials. The SST analog SMS 201-995 (octreotide, OCT) was obtained from Sandoz Pharma (Basel, Switzerland). D-Trp^8 SST-14 was purchased from Bachem (Torrance, CA). Reagents required for cellular staining and flow cytometry were procured from the following sources: Propidium iodide (PI) from ICN Pharmaceuticals (Costa Mesa, CA), Hoechst 33342 from Calbiochem (San Diego, CA) and, carboxy SNARF-1 and its cell permeant derivative aminomethyl ester from Molecular Probes (Eugene, OR). All other reagents were obtained from local commercial sources and were of analytical quality.

Cell lines. Establishment and characterization of CHO-K1 cell lines stably expressing individual hSSTR subtypes have been detailed elsewhere (13,14). Cells were grown in T75 flasks in Hams F-12 medium containing 5% fetal calf serum and 400 U/ml G-418 and cultured for 3-5 days at 37°C in a humidified atmosphere with 5% CO_2.

Preparation of nuclear protein extract and determination of endonuclease activity. Nuclear protein extract was prepared according to Eastman (9) from CHO-K1 cells expressing individual hSSTR subtypes pre-incubated with 100 nM peptides for 24 h. Briefly, 2×10^6 cells were resuspended and allowed to swell for 20 min on ice in buffer 20 mM Hepes-KOH buffer, pH 7.4 containing 0.75 mM spermidine, 0.15 mM spermine, 0.1 mM EDTA and, 1 mM each of DTT and phenylmethylsulfonyl fluoride (HSSE buffer). Cells were homogenized after adding sucrose to a final concentration of 0.5 M. This mixture was centrifuged for 10 min at 150 g, and the pellet was resuspended in 0.75 ml of the buffer containing 0.5 M sucrose, layered on 1.5 M sucrose/HSSE buffer and centrifuged for 20 min at 13,000 g. The nuclei were then resuspended in a buffer containing 10 mM sodium acetate, 10 mM sodium phosphate, 10 mM bistris-propane, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (APB buffer) and recentrifuged to remove residual spermine and spermidine. Nuclear protein extracts were prepared by resuspending the nuclei in APB buffer (pH 7) containing 0.5 M NaCl. After 1 h incubatuion on ice, the precipitated DNA was pelleted by centrifugation at 14,000 g for 10 min and the supernatant recovered.

The endonuclease assay was performed by incubating 100 ng of genomic DNA prepared from untransfected CHO-K1 cells as described previously (13) with 10 μg of nuclear protein extract in APB buffer adjusted to pH 5, 7 or 8 in the absence or presence of 10 mM Ca^{2+}/Mg^{2+}. The mixtures were incubated at 37° C for 30 min and electrophoresed on a 1.2% agarose gel (w/v) containing ethidium bromide and visualized under UV light.

Measurement of intracellular pH and apoptosis. Intracellular pH measurements were performed in cells preloaded with carboxy-SNARF-1 AM according to Barry et al. (7). hSSTR3 expressing cells were loaded with 10 μM acetoxymethylester derivative of SNARF-1 for during the final hour of the 24 h incubation in the absence or presence of 100 nM OCT at 37°C. The cells were then scraped, washed and resuspended in 2 ml of fresh medium and incubated for 1 min at 37°C with 1 μg/ml Hoechst 33342, a DNA binding dye which is taken up preferentially by apoptotic cells due to their altered membrane permeability (16). Cells were maintained at 37°C in a Becton-Dickinson FACStar Vantage cytometer. Hoechst 33342 was excited at 355 nm and its emission was measured at 440 nm. Intracellular carboxy SNARF-1 was excited at 488 nm and emission was recorded at both 580 and 640 nm with 5 nm band pass filters with linear amplifiers. The ratio of the emissions at these wavelengths was electronically calculated and used as a parameter indicative of intracellular pH. The intracellular pH values in control and treated cells were estimated by comparison of the mean ratios of the samples to a calibration curve of intracellular pH generated by incubation of carboxy-SNARF-1 loaded hSSTR3 expressing CHO-K1 cells in buffers ranging in pH from 8.0 to 6.25 and containing the proton ionophore nigericin. Data was analyzed using the WinList software (Verity Software Inc., Topsham, ME). Cells with fluorescence of <50 units were excluded in the calculation of the ratio of the emissions at 580 nm and 640 nm.

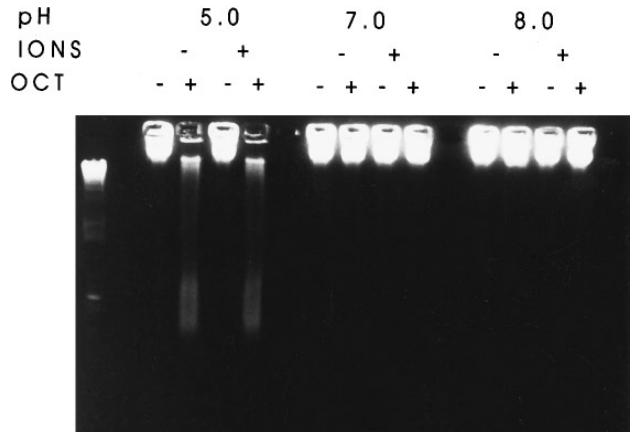


FIG. 1. Endonuclease activity in nuclear protein extracts of expressing hSSTR3 CHO-K1 cells incubated in the absence or presence of 100 nM OCT for 24 h was measured in vitro using the genomic DNA prepared from control CHO-K1. Following incubation for 15 min at 37C at pH 5,7 and 8 in the absence or presence of Ca^{2+} and Mg^{2+} as described under Methods, the samples were electrophoresed on 1.2% agarose gel, stained with ethidium bromide and visualized under UV light. Digestion of the DNA was observed only with nuclear protein extracts of OCT treated cells during incubation at pH 5 (lanes 2 and 4) but not in untreated cells (lanes 1 and 3). This enzyme activity was insenitive to divalent cations (compare lanes 2 and 4). By contrast, OCT did not induce Ca^{2+} and Mg^{2+} sensitive or insensitive endonucleases at pH 7 (lanes 5–8) or 8 (lanes 9–12). Data representative of 4 separate experiments.

RESULTS

Cytotoxic signaling of SST is selectively transduced through hSSTR3 and causes apoptosis characterized by the presence of oligonucleosome sized DNA fragments. In the present study we demonstrate that the DNA fragmentation in these cells is due to selective activation by OCT of a pH sensitive endonuclease which is active under acidic conditions (figure 1). The activity of this enzyme was not sensitive to Ca^{2+}/Mg^{2+} since the extent of DNA digestion did not increase in the presence of the cations. As illustrated in this figure, OCT did not induce endonucleases exhibiting neutral or alkaline pH optima either in the absence or presence of Ca^{2+}/Mg^{2+}. This endonuclease was not active at or above neutral pH, but increased as the pH was lowered progressively over a range of 6.7- 4.9 (figure 2). Its activity was maximal at the lowest pH tested. Activation of this endonuclease in CHO-K1 cells was clearly hSSTR subtype selective and occurred only via hSSTR3 (figure 3).

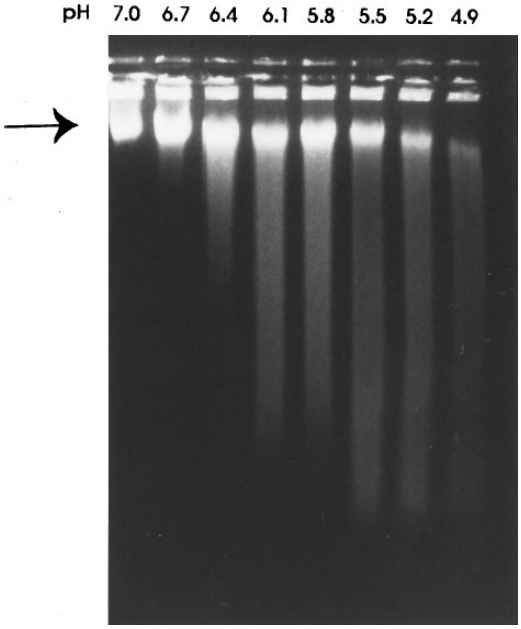


FIG. 2. Effect of pH on the activity of OCT induced acidic endonuclease. In vitro enzyme activity in nuclear protein extracts of OCT treated cells was measured in buffers ranging in pH from 7 to 5. Maximum activity was observed at the lowest pH tested. Data representative of 2 separate experiments.

In cells expressing hSSTR3, OCT induced pH dependent endonuclese activity increased in a time dependent manner. When tested for its ability digest DNA in vitro, its activity was detectable after 1 h during OCT treatment (figure 4A). This is evident from the decrease in the intensity of the labeling of intact DNA (which remains close to the origin, lane 2), the marked increase in the intesntiy of the digested DNA fragments at 4 h (lane 3) followed by

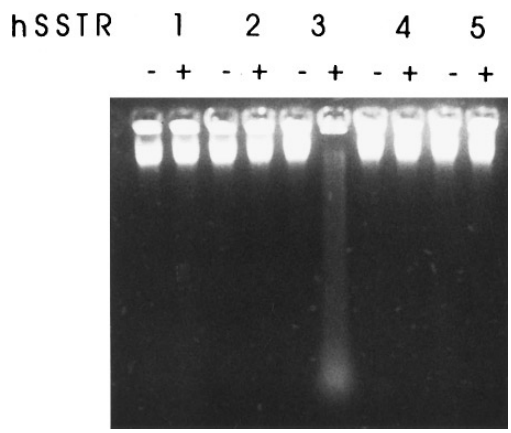


FIG. 3. Subtype selectivity of hSSTR signaled activation of endonuclease II. Cells were incubated in the absence or presence of 100 nM OCT (hSSTRs 2,3,5) or 100 nM D-Trp^8 SST-14 (hSSTRs 1,4) for 24 h. The endonuclease activity in nuclear extracts was assessed at pH 5. Data representative of 4 separate experiments.

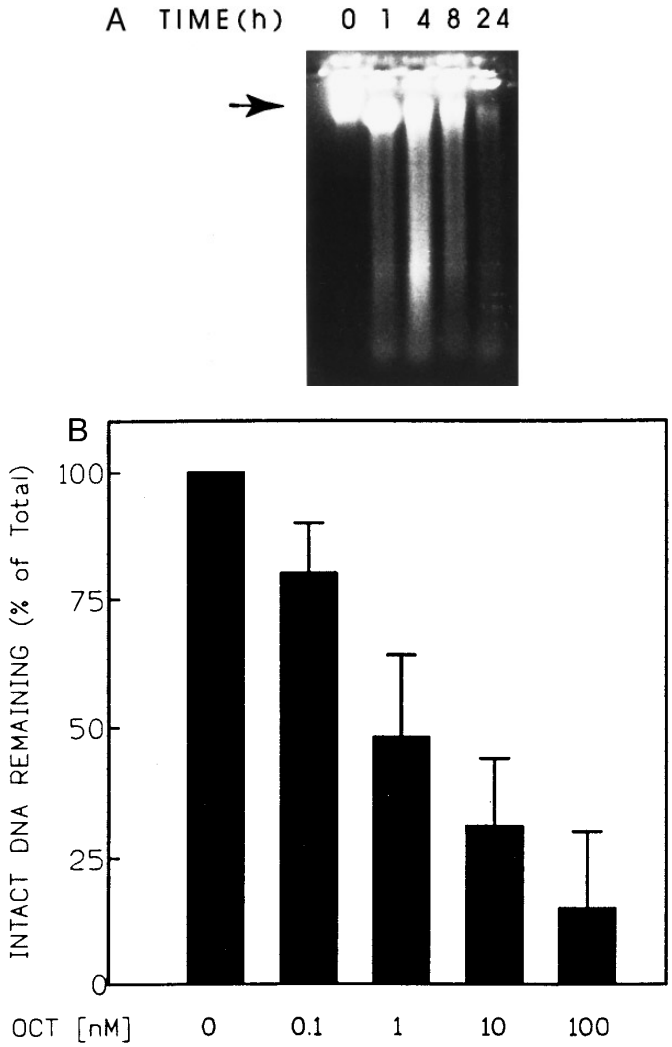


FIG. 4. Time dependent activation of acidic endonuclease by OCT (100 nM) in CHO-K1 cells expressing hSSTR3. **A.** Representative agarose gel electrophoresis demonstrating the time dependent decrease in the intensity of the intact DNA indicated by the arrow. Notice also that he intensity of ethidium bromide staining of DNA fragments increases initially reaching a maximum by 4 h, but decreases at later time points. **B.** Time dependent activation of this endonuclease by OCT was quantitated by densitometric measurement of intact DNA remaining at each time point and expressed as a percentage of the DNA in untreated cells (time 0). values represent mean ± SE, n=4.

decrease in both the intact and fragmented DNA at longer time points of 8 and 24 h (lanes 4,5). The effect of OCT on the activity of this enzyme was quantitated densitometrically by measuring the decrease in the intensity of the labeling of the intact DNA. The decrease in the intensity of ethidium bromide statining of the intact DNA band at 1 h was 12 ± 6% and was 88 ± 15 % at 24 h (figure 4B). hSSTR3 signaled activation of this endonuclease by OCT was dependent on its concentration over a range of 0.1-100 nM (figure 5 A,B). The loss of intensity of the intact DNA

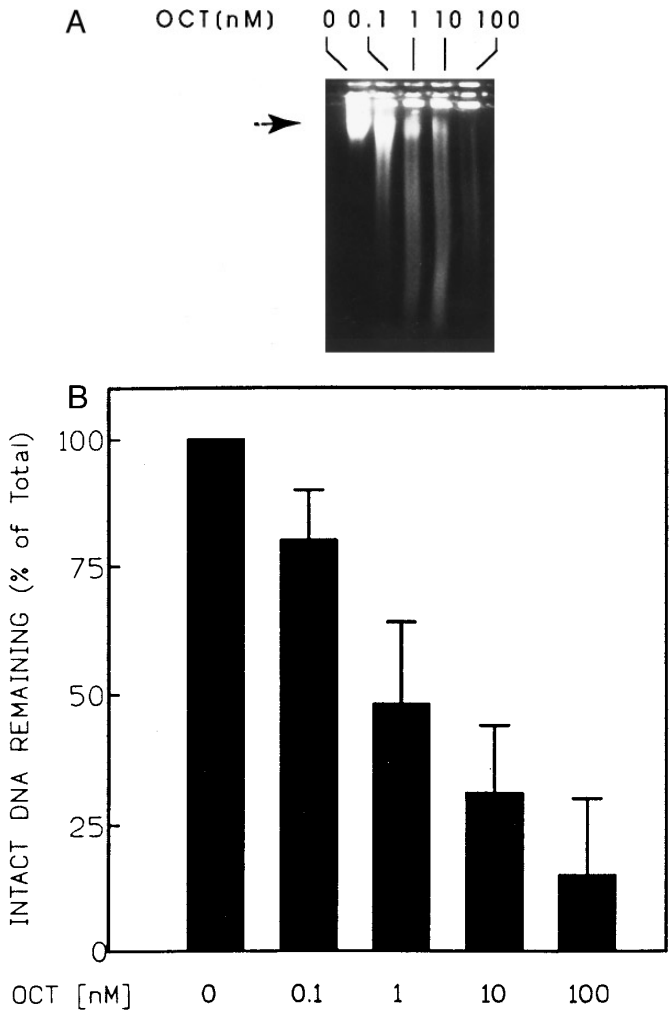


FIG. 5. Dose-dependent activation of acidic endonuclease by OCT in CHO-K1 cells expressing hSSTR3. **A.** Representative agarose gel electrophoresis showing the concentration dependent activation by OCT of the endonuclease activity Arrow indicates the intact DNA. **B.** The intact DNA remaining at each OCT concentration was quantitated densitometrically and expressed as a percentage of intact DNA in untreated cells. Values represent mean ± SE, n=4.

ranged from 20 ± 10 % to 82 ± 15 % respectively in cells treated with 0.1 and 100 nM OCT.

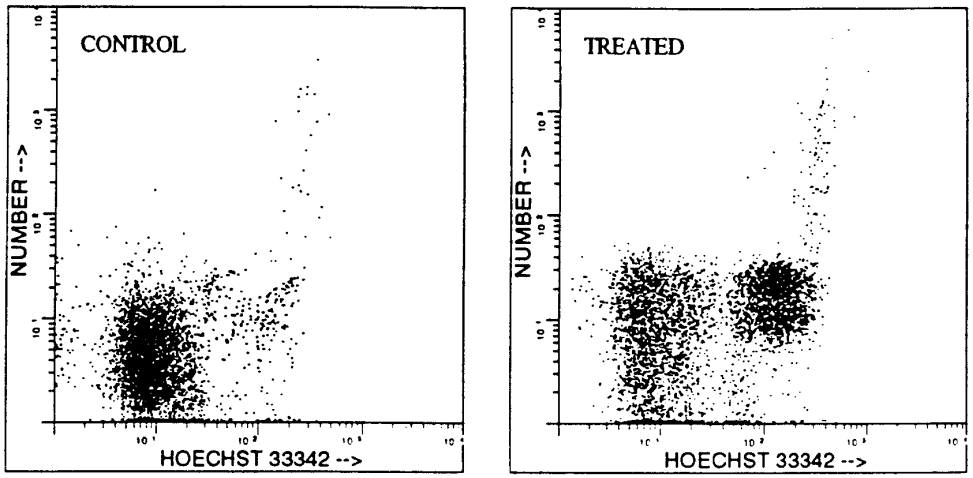
The above finding of involvement of a pH dependent acidic endonuclease in OCT signaled apoptosis suggested that intracellular acidification may underly cytotoxic signaling via hSSTR3. To test this, we measured the intracellular pH using the dye carboxy SNARF-1 in cells undergoing OCT induced apoptosis. Apoptotic cells were characterized by their increased Hoechst 33342 fluorescence following 24 h treatment with the peptide (figure 6A, right panel) while untreated control cells displayed low dye uptake (figure 6A, left panel). Apoptic cells exhibiting increased Hoechst 33342 averaged 34 +5% (n=3). Intracellular pH was measured by analysing the carboxy-SNARF-1 fluorescence excited at 488 nm and the emission recorded at 585 vs 640 nm wavelengths. The data is presented as a two dimensional dot plot with 580 nm fluorescence on the abscissa and the 640 nm fluorescence on the ordinate. Control cells, not treated with the peptide, were found distributed along a single line out of the origin (figure 6B, left panel). Since the distance of each cell from the origin is directly proportional to the amount of carboxy-SNARF-1 loading, these cells possess the same intracellular pH. By contrast, OCT treatment led to the appearance of a distinct population of cells with a increased fluorescence at 580 nm and shifting to the right indicative of lower intracellular pH (figure 6B, right panel). The decrease in pH in these cells was determined by comparing the ratio of emissions at these wavelengths against a standard curve generated from the emission ratios at 580 and 640 nm in carboxy-SNARF-1 loaded cells which were incubated in buffers ranging in pH from 6.25 - 8.0 in the presence of the proton ionophore nigericin. The recordings obtained in cells incubated at buffers at pH 8 and 6.25 containing nigericin are compared in figure 6C (left panel). A calibration curve was generated from the ratios of emissions at 580 nm and 640 nm obtained in the different buffers containing nigericin (figure 7C, right panel). The intracellular pH was 6.5 ± 0.2 in OCT treated cells compared to 7.2 ± 0.1 in untreated, control cells (n=3) (figure 7). The intracellular pH was 6.3 ± 0.16 in cells exhibiting increased Hoechst 33342 fluorescence. The apoptic cells were thus even more acidic. OCT induced decrease in cellular pH as well as the induction of the acidic endonuclease was inhibited by orthovanadate and abolished by pertussis toxin pretreatment (details not shown). In cells expressing other hSSTRs which did not signal apoptosis nor stimulate endonuclease activity, no change in intracellular pH occurred during agonist treatment.

DISCUSSION

In the present study, we demonstrate that hSSTR3 mediated cytotoxic signaling is associated with selective activation of a pH dependent endonuclease. This novel finding indicates that the endonuclease responsive to OCT is active under acidic conditions and is Ca^{2+}/Mg^{2+} insensitive. This enzyme thus appears to be similar to DNAse II previously implicated in CHO cell apoptosis induced by a variety of cytotoxic drugs (7–9). OCT did not induce cation sensitive endonucleases that are known to be active at neutral or alkaline pH and implicated in other models of apoptosis (3–6).

Activation of a pH dependent, cation insensitive endonuclease during hSSTR3 signaled apoptosis suggested that intracellular acidification may be an underlying cause. Using carboxy-SNARF-1 we demonstrate that OCT induced apoptosis is indeed associated with intracellular acidification. Such acidification was lim-

A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HOECHST 33342 -->
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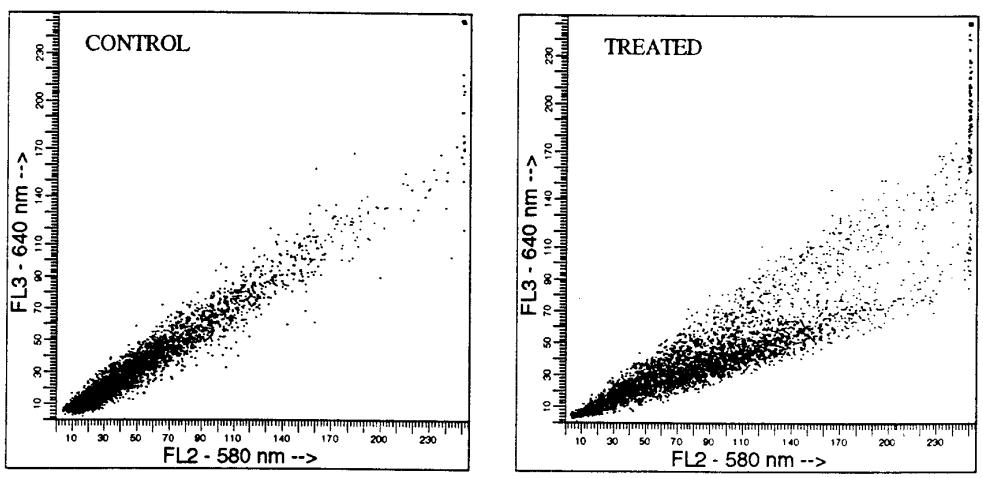
B


CONTROL
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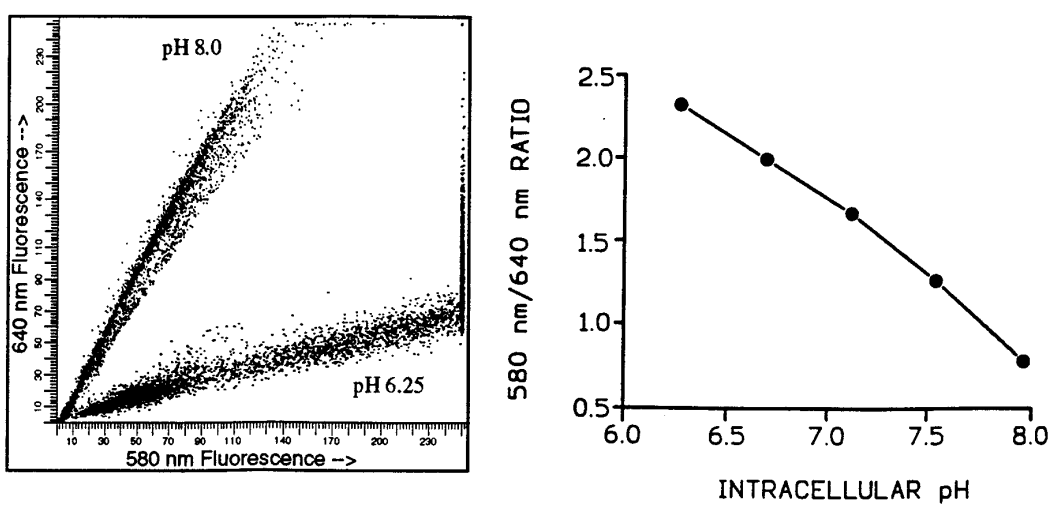
TREATED
FL3 - 640 nm -->
FL2 - 580 nm -->

C


pH 8.0
pH 6.25
640 nm Fluorescence -->
580 nm Fluorescence -->
580 nm/640 nm RATIO
INTRACELLULAR pH

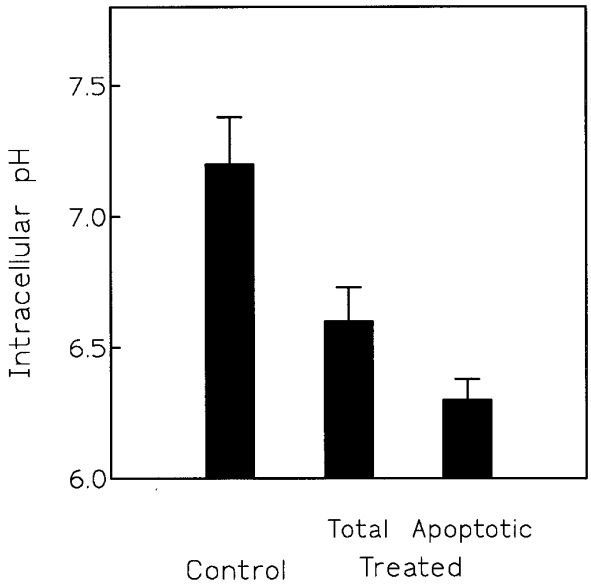


FIG. 7. OCT induced decrease in intracellular pH was greater in apoptotic (Hoechst 33342 positive) cells (6.3 ± 0.1) compared to the treated cells taken as a whole (6.5 ± 0.2). The pH of untreated cells was 7.2 ± 0.1. (mean ± SE, n=3)

ited to the cells that became Hoechst 33342 positive. This suggests that intracellular pH decrease may occur only in cells undergoing apoptosis induced by OCT. This is similar to the decrease in intracellular pH observed in other models of apoptosis involving activation of acidic endonuclease (7,10,11). The pH in these cells averaged 6.6 ± 0.2, a pH at which the endonuclease activity is detectable, but not maximal, under in vitro conditions (figure 2). We have shown earlier that Bax but not Bcl-2 is induced during hSSTR3 signaled apoptosis (13). Bax as well as Bcl-2 can form pH-dependent channels but the intrinsic properties of channels formed by these proteins differ with respect to pH (16,17). In planar bilipid layers, Bax can form pH and voltage dependent ion-conducting channels at neutral and acidic pH whereas Bcl-2 is effective only at a pH $<$5.0 (17). The selective activation of Bax by OCT via hSSTR3 may therefore account, in part, for the decrease in intracellular pH in these cells. Additional mechanisms involving Na^+/H^+ antiporter and H^+-ATPase can also trigger cellular acidification For instance, in HeLa cells and in murine hematopoeitic cells, inhibition of Na^+/H^+ antiporter activity has been shown to decrease intracellular pH and activate acidic endonuclease (18,19). Furthermore, pharmacological induction of Na^+/H^+ antiporter can prevent acidification and endonuclease activation (19). The potential involvement of Na^+/H^+ antiporter in hSSTR3 initiated cytotoxic signaling needs to be assessed.

The present findings constitute, to our knowledge, the first instance of a G protein coupled receptor agonist induced intracellular acidification and activation of an acidic endonuclease leading to apoptosis Furthermore, the cytotoxic effect is uniquely signaled via a single SSTR subtype, namely hSSTR3. Both PTx pretreatment and orthovanadate abolished OCT induced pH decrease and activation of the acidic endonuclease. Another G protein coupled receptor AT_2R has recently been reported to initiate apoptosis in neonatal ventricular myocytes (20). Angiotensin II acting via AT_2R appears to signal in a different manner in ventricular myocytes by elevating intracellular Ca^{2+} and activating a calcium dependent endonuclease, but not intracellular acidification (20).

In CHO-K1 cells expressing the other four hSSTRs agonist treatment did not alter intracellular pH. Additionally, no induction of endonuclease activity was observed in these cells during agonist treatment (details not shown). This is in accord with our earlier finding that these hSSTRs are incapable of eliciting a cytotoxic response, although agonist treatment inhibits cell growth via other human SSTR subtypes (13,21). How can hSSTR3 but not the other hSSTR subtypes transduce cytotoxic signaling? The five hSSTRs exhibit most homology in the transmembrane domains (55-70% sequence identity) and share considerable sequence identity (38-54%) in regions comprising the second and third intracellular loops, the putative G protein binding region in G protein coupled receptors (22). The cytoplasmic tail (C-tail) sequence of hSSTR3 however differs markedly from that of the four other subtypes (12-18% sequence identity, reviewed in 23) suggesting that the C-tail of hSSTR3 may be the functional domain

FIG. 6. hSSTR3 signaled apoptosis in CHO-K1 cells is associated with intracellular acidification. **A.** Apoptotic cells were identified by their increase uptake of Hoechst 33342. In control cells there is minimal uptake of the dye as indicated by the low fluorescence (left panel) whereas 40% of the cells become apoptotic following 24 h treatment with 100 nM OCT and display $>$10 fold higher Hoechst 33342 fluorescence (right panel, upper quadrant). **B.** Effect of OCT on carboxy-SNARF-1 fluorescence. Following incubation in the absence or presence of 100 nM OCT for 24 h, cells were loaded with carboxy-SNARF-1 and cells were excited at 488 nm and fluorescence measured at 585 and 640 nm in a Becton-Dickinson Vantage Plus flow cytometer as described under Methods. When the fluorescence at 580 nm is plotted against that at 640 nm, cells distributed along the same line out of the axis possess the same pH as seen in untreated cells since fluorescence intensity of the cells is directly proportional to the dye uptake (left panel). Following OCT treatment, ~40% of the cells display an increase in fluorescence at 580 nm relative to the fluorescence at 640 nm which is reflected by the downward shift in the distribution of the cells (right panel). **C.** Generation of a pH calibration curve. Carboxy-SNARF-1 loaded cells were incubated in the presence of the proton ionophore nigericin in buffers ranging in pH from 6.25 - 8. Representative plots of cells incubated at pH 8 and 6.25 are shown (left panel). A pH calibration curve was derived from the ratios of the emissions at 585 and 640 nm (right panel). The intracellular pH of CHO-K1 cells was 7.2 ± 0.1 (n=3). OCT induced acidification was reflected by the decrease in pH to 6.5 ± 0.2 (n=3), a loss of 0.7 pH units.

that confers specificity for apoptotic signaling. Studies to test this directly by mutagenesis are currently in progress.

The cytotoxic signaling via hSSTR3, which leads to intracellular acidification, activation of an acidic endonuclease and apoptosis, is PTx-sensitive G protein mediated. Other G protein coupled receptors such as the angiotensin type II receptor (AT_2R) and dopaminargic D_2 receptors (D_2R) also transduce antiproliferative signaling in PTx-sensitive G protein dependent manner (24,25). PTx-sensitive G protein dependent induction of apoptosis has also been observed with T cell and B cell receptors (TCR and BCR) which interact with distinct antigens and initiate cellular responses primarily by triggering tyrosine kinases (26,27). Additionally, activation-induced cell death mediated through simultaneous ligation of CD16/IL-2 receptor in human natural killer cells is prevented by PTx pretreatment (28). Only the negative consequnces of TCR-mediated stimulation, namely apoptosis, is inhibited by PTx while the positive consequences including calcium mobilization, lymphokine secretion and proliferation remain unaffected (28,29). Whether these receptors which belong to different receptor family interact directly with G proteins and initiate cytotoxic signaling or require activation of a G protein coupled receptor during cytotoxic signaling is not known. In this context, it is of interest that induction of a putative G protein coupled receptor TDAG8 has been reported to be associated with T cell apoptosis signaled by TCR mediated activation as well as by glucocorticoids (30).

In summary, the present findings demonstrate that SST signaled apoptosis is associated with the activation of an acdic, cation insensitive endonuclease and intracellular acidification. These events are signaled in a subtype selective manner uniquely via hSSTR3.

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