In nonexcitable cells, stimulation by high agonist concentrations typically produces a biphasic increase in cytosolic Ca^{2+} ([Ca^{2+}]_i). This response is characterized by a transient initial increase because of intracellular Ca^{2+} release followed by a sustained elevation which varies in amplitude depending on the nature of the stimulus. In contrast, low-level stimulation often evokes oscillatory changes in [Ca^{2+}]_i. The specific information provided by repetitive [Ca^{2+}]_i spikes appears to be encoded in the frequency rather than in the amplitude of [Ca^{2+}]_i oscillations. The specific, membrane-permeable inositol 1,4,5-trisphosphate (Ins-1,4,5-P_3) receptor blocker Xestospongin C (XeC) (16) was used to examine the role of [Ca^{2+}]_i oscillation frequency in the regulation of NF-κB-driven gene expression in human aortic endothelial cells (HAEC) during low-level histamine stimulation.

We found that a decrease in the frequency of [Ca^{2+}]_i oscillations during low-level histamine stimulation resulted in a parallel decrease in NF-κB activity. The regulation of nuclear transcriptional activity by the frequency of cytosolic Ca^{2+} oscillations may provide cells with a specific mechanism to control gene expression during agonist stimulation.

**MATERIALS AND METHODS**

**Cell Culture and [Ca^{2+}]_i Measurement—HAEC (Clonetics) were grown to passage 5–9 at ~70% confluence on gelatin-coated, 25-mm diameter circular glass coverslips (VWR Scientific) (7). HAEC [Ca^{2+}]_i, was measured after loading with 10 μM concentration of the acetoxymethyl ester form of the low-affinity Ca^{2+}-bound and Ca^{2+}-free forms of the indicator, respectively. The intracellular minimum and maximum concentrations ([R]_min and [R]_max, respectively) were determined as described previously (17), and these were used to calculate [Ca^{2+}]_i, according to the following formula: [Ca^{2+}]_i = [R]_max – [R]_min/[R]_max – R[S]/[S]_0 (18), where [K]_d is the dissociation constant of indo 1, and [S]_0 and [S]_2 are the fluorescence intensities at ~490 nm of the Ca^{2+}-free and Ca^{2+}-saturated indicator, respectively. [K]_d was determined to be 207 nm under the present experimental conditions using an in vitro calibration method (17). In some experiments, XeC (2, 6, and 20 μM, Calbiochem) was used after being dissolved in 0.01–0.1% v/v dimethyl sulfoxide (Me_2SO, Sigma).

**Ca^{2+} Release Measurement—Endoplasmic reticulum (ER) Ca^{2+} concentration was monitored in saponin- (30 μg/ml, Sigma) permeabilized HAEC after loading with 10 μM concentration of the acetoxymethyl ester form of the low-affinity Ca^{2+} indicator, Mag-indo 1 (Molecular Probes) (19, 20), for 45 min at room temperature. Cells were then exposed for 2–3 min to Mg^{2+}/ATP-free intracellular-like medium (ICM) containing (in mM): 125 KCl, 19 NaCl, 10 HEPES, 1 EGTA (Sigma) 1, and 0.33 CaCl_2 (free Ca^{2+} concentration = 50 nM, pH adjusted to 7.20 at room temperature with KOH). After permeabilization, HAEC were washed with Mg^{2+}/ATP-free ICM and then exposed to complete ICM (containing 1 mM ATP and 1.4 mM MgCl_2, free Mg^{2+} concentration = 0.1 mM) for 10 min to allow for filling of intracellular Ca^{2+} stores. Permeabilized HAEC were then superfused for at least 10 min with Ca^{2+}-
releasing medium (CRM) containing (in mM): 125 KCl, 19 NaCl, 10 HEPES, 1.4 MgCl2, and 150 mM CaCl2 (calculated concentration confirmed by fluorescence of indo 1 free acid). The pH of the CRM was adjusted to 7.20 with KOH. Mag-indo 1 fluorescence was recorded at room temperature from single HAEC on coverslips in a perfusion chamber mounted on the stage of a modified Nikon Diaphot inverted epifluorescence microscope as described above for indo 1. Since low-affinity Ca2+-sensitive indicators like Mag-indo 1 report free Ca2+ concentration in the micromolar range and accumulate in intracellular organelles, they may be effectively used to monitor the free Ca2+ content in these organelles but are relatively insensitive to changes in cytosolic Ca2+, which typically occur in the nanomolar range.

The fluorescence ratios at zero and saturating free calcium were determined from 10 μM Mag-indo 1 free acid solutions in Mg2+/ATP-free ICM containing no added CaCl2 and 1 mM EGTA or containing 10 mM CaCl2, respectively, and were used as the minimum and maximum ratios (Rmin and Rmax, respectively). Rmin = 0.19 ± 0.04 and Rmax = 4.56 ± 0.75 for an average of six experiments using Mag-indo 1-loaded HAEC. The approximate ER Ca2+ was calculated as described previously (18, 20), according to the formula Ca2+ = Kd(R - Rmin)/(Rmax - R(S1/S0)), where Kd is the dissociation constant of Mag-indo 1 and S1 and S0 are the fluorescence intensities at 490 nm of the Ca2+-free and Ca2+-saturated indicator, respectively. When CRM with different concentrations of free Ca2+(pCa from 8.0 to 5.0) were used, the free Ca2+ concentration was buffered using 10 mM EGTA and 10 mM Ca-EGTA as described previously (21, 22).

Transfection and Chloramphenicol Acetyltransferase (CAT) Assays—An NF-κB-CAT reporter plasmid was transfected into HAEC using LipofectAMINE (Life Technologies, Inc.) as described previously (23). Briefly, HAEC were seeded in 60-mm dishes (Falcon) at ~2 × 106/ml. After reaching ~70% confluence, cells were incubated with 2 μg of the reporter plasmid and 12 μg of LipofectAMINE per dish. Cells were harvested 72 h after transfection and CAT activity was assessed using [14C]chloramphenicol (NENTM Life Science Products, Inc.) and a CAT enzyme assay system (Promega). CAT activity was normalized to the total number of HAEC. The results of three separate experiments using HAEC containing the NF-κB-CAT reporter were used to report CAT activity after stimulation with 1 μM histamine in the absence or presence of XeC.

RESULTS

XeC Decreases the Frequency of [Ca2+], Oscillations during Histamine Stimulation—[Ca2+]i oscillations occurred in 26 of 33 HAEC stimulated by 1 μM histamine. The amplitude of these oscillations was relatively constant in 24 of these 26 HAEC (Fig. 1A). In 4 of the 33 HAEC studied, a single [Ca2+]i transient was observed, and 3 showed no response to this concentration. At higher concentrations (10 μM) histamine typically stimulated a biphasic increase in [Ca2+]i, characterized by a transient initial increase followed by a persistent elevation above baseline (not shown). [Ca2+]i oscillations were inconsistently observed (4 of 11 cells) at a lower concentration of histamine (0.3 μM); the remainder exhibited single [Ca2+]i transients or no response.

During an established response to 1 μM histamine, characterized by sustained, nondecremental [Ca2+]i oscillations, XeC produced a dose-dependent decrease in [Ca2+]i oscillation frequency without affecting oscillation amplitude (Fig. 1B). There was a trend for 2 μM XeC to decrease [Ca2+]i oscillation frequency (83.6 ± 5.4%, p = NS versus control, Fig. 1C), but this did not achieve statistical significance until a concentration of 6 μM (63.1 ± 8.6%, p < 0.05). At a concentration of 20 μM, XeC further decreased [Ca2+]i oscillation frequency (45.9 ± 7.4%, p < 0.05 versus 2 μM XeC and versus control). The effect of XeC on the frequency of [Ca2+]i oscillations was partially reversible after washout, as [Ca2+]i oscillation frequency recovered to 68.0 ± 7.9% (p < 0.05 versus 20 μM XeC). No changes in [Ca2+]i oscillation amplitude were observed during exposure to XeC (912.3 ± 53.7 nm for control versus 953.5 ± 127.4 nm for 20 μM XeC, p = NS).

XeC Is a Noncompetitive Inhibitor of the InsP3 Receptor—The mechanism of the effect of XeC on [Ca2+]i oscillation frequency was examined in permeabilized HAEC using the low-affinity Ca2+ probe Mag-indo 1 to monitor ER Ca2+ release. When intact HAEC monolayers were loaded with Mag-indo 1, fluo-
rescence of the dye distributed uniformly throughout the cytoplasm. Saponin permeabilization caused a large loss of intracellular fluorescence because of membrane permeabilization and release of intracellular dye. The remaining fluorescence appeared visually to encircle the nucleus. Over a 25-min period, fluorescence intensity decreased by 15% (F405:14.5 ± 2.4%, F485:15.9 ± 3.2%), although the F405:F485 fluorescence ratio changed by 1%. When permeabilized HAEC were exposed to Mg2+- and ATP-containing medium to allow for filling of intracellular Ca2+ stores, the increase of the Mag-indo 1 ratio was dependent on the Ca2+ concentration in the presence of Mg2+ and ATP but was not affected by either Mg2+ alone, Mg2+ and ATP, or Ca2+ alone. These findings are consistent with the known characteristics of Mag-indo 1, whose sensitivity to Mg2+ is 100-fold less than to Ca2+ (Kd = 3.1 mM for Mg2+ and 35 μM for Ca2+ both at 22 °C, pH = 7.40) (24). The addition of Ins-1,4,5-P3 stimulated a concentration-dependent decrease in ER Ca2+ that saturated at 1 μM (Fig. 2A). The subsequent

**Fig. 2. Effect of XeC on InsP3-induced endoplasmic reticulum (ER) Ca2+ release.** A, representative tracing of five similar experiments from a Mag-indo 1-loaded single permeabilized HAEC after ER Ca2+ filling. The addition of Ins-1,4,5-P3 (0.1–3 μM) stimulated dose-dependent ER Ca2+ release, as evidenced by a decrease in the Mag-indo 1 fluorescence ratio. The effect of Ins-1,4,5-P3 saturated at 1 μM, but 1 μM ionomycin released an additional Ins-1,4,5-P3-insensitive intracellular Ca2+ store. Vehicle (0.01–0.1% Me2SO) was present throughout. B, representative tracing of three experiments from a Mag-indo 1-loaded single permeabilized HAEC in the presence of 20 μM XeC (open triangle). XeC inhibited the effect of Ins-1,4,5-P3 on ER Ca2+ release in comparison to vehicle control but did not affect the response to ionomycin. C, averaged data showing that XeC produced dose-dependent inhibition of Ins-1,4,5-P3-induced Ca2+ release. Ins-1,4,5-P3-induced Ca2+ release is expressed as a percentage of that induced by 1 μM ionomycin in each HAEC studied (data represent mean ± S.E. of three experiments for each group).
addition of 1 μM ionomycin further emptied ER Ca\(^{2+}\) stores. In the presence of 20 μM XeC (Fig. 2B), Ins-1,4,5-P\(_3\)-induced Ca\(^{2+}\) release was inhibited, but the response to ionomycin was unaffected. XeC (6 and 20 μM) inhibited the maximal ER Ca\(^{2+}\) release induced by Ins-1,4,5-P\(_3\) without affecting the approximate EC\(_{50}\) for Ins-1,4,5-P\(_3\) (440.2 ± 51.0 nM for 6 μM XeC and 483.7 ± 41.7 for 20 μM XeC, p = NS versus vehicle control, 423.2 ± 54.9 nM, Fig. 2C).

The effect of a submaximal concentration of Ins-1,4,5-P\(_3\) (300 nM) on ER Ca\(^{2+}\) was studied at different Ca\(^{2+}\) concentrations (pCa 8.0 to pCa 5.0) to further characterize the mechanism of XeC. XeC stimulated a dose-dependent shift to the right of the ER Ca\(^{2+}\) release-pCa relationship (Fig. 3). Increasing the Ca\(^{2+}\) concentration gradually increased Ins-1,4,5-P\(_3\)-induced Ca\(^{2+}\) release from 2.6 ± 0.6% at pCa 8.0 to a maximal effect of 55.7 ± 2.2% for pCa 6.5. Further increases in Ca\(^{2+}\) concentration gradually increased Ins-1,4,5-P\(_3\)-induced Ca\(^{2+}\) release (28.8 ± 4.2%, 16.6 ± 3.0%, and 5.9 ± 1.5% for pCa 6.0, 5.5, and 5.0, respectively); this is similar to the bell-shaped ER Ca\(^{2+}\) release-pCa relationship reported previously (22). In the presence of 20 μM XeC, the Ca\(^{2+}\) concentration for maximal ER Ca\(^{2+}\) release induced by Ins-1,4,5-P\(_3\) increased from a pCa of 6.5 to a pCa of 6.0. Thus, in the presence of XeC, higher Ca\(^{2+}\) concentrations are required to activate Ins-1,4,5-P\(_3\) receptors than in the absence of XeC. Together, these results show that XeC produces a dose-dependent decrease in the frequency of established [Ca\(^{2+}\)]\(_i\) oscillations by noncompetitive inhibition of the Ins-1,4,5-P\(_3\) receptor.

XeC Decreases NF-κB-CAT Reporter Activity during Histo-

amine Stimulation—HAEC were transfected with an NF-κB-

CAT reporter plasmid (23) to determine whether histamine stimulates NF-κB transcriptional activity in HAEC, as it does in epithelial cells (15). Histamine (1 μM) induced an ~14-fold increase in reporter gene activity from a basal level of 0.05 ± 0.00 to 0.70 ± 0.08 units/100 μl of extract/3 × 10\(^5\) cells, Fig. 4). XeC, at concentrations that decrease [Ca\(^{2+}\)]\(_i\), oscillation frequency in histamine-stimulated HAEC, also decreased CAT reporter activity in HAEC stimulated with 1 μM histamine. This effect of XeC, at concentrations that decrease [Ca\(^{2+}\)]\(_i\), oscillation frequency in histamine-stimulated HAEC, also decreased CAT reporter activity in HAEC stimulated with 1 μM histamine (Fig. 4). XeC, at concentrations that decrease [Ca\(^{2+}\)]\(_i\), oscillation frequency in histamine-stimulated HAEC, also decreased CAT reporter activity in HAEC stimulated with 1 μM histamine (Fig. 4).

We employed the recently developed membrane-permeable Ins-1,4,5-P\(_3\)-receptor blocker XeC to dose-dependently decrease the frequency of established [Ca\(^{2+}\)]\(_i\), oscillations during low-level histamine stimulation. At a concentration of 20 μM, XeC decreased the frequency of [Ca\(^{2+}\)]\(_i\) oscillations and NF-κB transcriptional activity each by more than 50%. This effect of XeC on Ca\(^{2+}\) signaling results from noncompetitive inhibition of the Ins-1,4,5-P\(_3\) receptor and a change in its Ca\(^{2+}\) sensitivity. At the concentrations used in this study, XeC decreased the frequency of histamine-stimulated [Ca\(^{2+}\)]\(_i\) oscillations within the oscillation frequency dependence range of NF-κB activity previously reported in T lymphocytes in which a “calcium clamp” method was used to generate [Ca\(^{2+}\)]\(_i\) oscillations in the absence of agonist stimulation (21). The use of XeC to affect Ca\(^{2+}\) signaling establishes, for the first time, a direct link between [Ca\(^{2+}\)]\(_i\), oscillation frequency and the activity of the nuclear transcription factor NF-κB during agonist stimulation.

Inactive NF-κB is present in the cytosol, complexed with the inhibitory protein, IκB. Disinhibition of NF-κB occurs when it is released from IκB inhibition, resulting in the translocation of NF-κB to the nucleus and the binding of NF-κB to the κB motif of the target gene (25). The release of active NF-κB is Ca\(^{2+}\)-dependent since [Ca\(^{2+}\)]\(_i\) elevations activate NF-κB (26), whereas NF-κB activity is decreased by buffering [Ca\(^{2+}\)]\(_i\) (26) or by inhibiting Ca\(^{2+}\) influx (27). It has been thought that Ca\(^{2+}\)-dependent processes like NF-κB activation are stimulated when their threshold [Ca\(^{2+}\)]\(_i\) for activation is exceeded. If this view is correct, it is unclear how a given stimulus which increases [Ca\(^{2+}\)]\(_i\), above the threshold for activation of many intracellular targets can activate some but not others. It has recently been suggested that [Ca\(^{2+}\)]\(_i\) oscillations may confer signaling specificity by their frequency rather than by the amplitude of repetitive [Ca\(^{2+}\)]\(_i\) spikes (8–10). Frequency modulation may enhance signaling efficiency and permit cells to respond to physiologic stimuli even when peak [Ca\(^{2+}\)]\(_i\) only periodically exceeds the threshold for activation. The regulation of nuclear transcriptional activity by [Ca\(^{2+}\)]\(_i\) oscillation frequency as demonstrated in the present study may provide cells with a specific mechanism whereby gene expression is controlled during agonist stimulation.

REFERENCES


DISCUSSION

We employed the recently developed membrane-permeable Ins-1,4,5-P\(_3\)-receptor blocker XeC to dose-dependently decrease the frequency of established [Ca\(^{2+}\)]\(_i\), oscillations during low-level histamine stimulation. At a concentration of 20 μM, XeC decreased the frequency of [Ca\(^{2+}\)]\(_i\) oscillations and NF-κB transcriptional activity each by more than 50%. This effect of XeC on Ca\(^{2+}\) signaling results from noncompetitive inhibition of the Ins-1,4,5-P\(_3\) receptor and a change in its Ca\(^{2+}\) sensitivity. At
[Ca^{2+}]_i Oscillation Frequency Regulates Agonist-stimulated NF-κB Transcriptional Activity
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doi: 10.1074/jbc.274.48.33995

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