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# Cytosolic calcium changes in endothelial cells induced by a protein product of human gliomas containing vascular permeability factor activity

GREGORY R. CRISCUOLO, M.D., PETER I. LELKES, PH.D., DANIEL ROTROSEN, M.D., AND EDWARD H. OLDFIELD, M.D.

*National Institute of Neurological Disorders and Stroke (Surgical Neurology Branch), National Institute of Diabetes and Digestive and Kidney Diseases (Laboratory of Cell Biology and Genetics), and National Institute of Allergy and Infectious Disease (Laboratory of Clinical Investigation), Bethesda, Maryland*

A vascular permeability factor (VPF) derived from serum-free conditioned medium of cultured human malignant gliomas (HG-VPF) has been described previously. The rapid kinetics of HG-VPF activity in an *in vivo* assay of vascular permeability suggests a direct action upon the vascular endothelial cell. To determine whether HG-VPF was capable of inducing a physiologically significant alteration in isolated endothelial cells, cytosolic calcium $[Ca^{++}]_i$ was measured *in vitro* in these cells before and after their exposure to media containing this substance. This was accomplished by preloading cultured endothelial cells with a fluorescent intracellular $Ca^{++}$ probe fura-2/AM.

It was found that HG-VPF induced a rapid and transient elevation of $[Ca^{++}]_i$ in normal endothelial cells derived from human umbilical vein, bovine adrenal medulla, bovine pulmonary artery, and rat brain. This effect was inhibited by chelating extracellular calcium $[Ca^{++}]_e$ with ethyleneglycol-bis ($\beta$-aminoethylether)-N,N'-tetra-acetic acid (EGTA), indicating that the HG-VPF-induced response resulted from the influx of extracellular calcium. The addition of cations that act as nonspecific calcium channel blockers ($Li^+$, $Co^{++}$, $Mn^{++}$, $La^{+++}$) completely inhibited VPF activity, further supporting the role of $[Ca^{++}]_e$ influx. The HG-VPF activity was not, however, blocked by verapamil, a calcium antagonist that appears to be specific for voltage-gated calcium channels. Furthermore, exposure of endothelial cells to 120 mM $[K^+]_e$ did not result in a calcium transient. Coincubation of endothelial cells with dexamethasone inhibited HG-VPF-induced rises in $[Ca^{++}]_i$, while having no effect upon cyclic nucleotide-induced changes in calcium.

The present studies indicate that vascular extravasation induced by human glioma-derived VPF may be mediated by a direct action upon vascular endothelial cells. Furthermore, the observed dexamethasone-induced inhibition of this process suggests a specific cellular action for corticosteroids. This, together with previous observations that dexamethasone suppresses both the production of VPF by tumor cells *in vitro* and its permeability-inducing activity *in vivo*, may explain the efficacy of glucocorticoids in the treatment of neoplastic vasogenic brain edema.

Finally, studies with a polycationic peptide (protamine) known to induce blood-brain barrier disruption *in vivo* revealed similar effects upon endothelial cytosolic calcium levels. As HG-VPF is a positively charged macromolecule, a common interaction between these substances and the negatively charged endothelial cell surface in the induction of permeability is suggested. Nonspecific cross-linking of charged groups of the endothelial glycocalyx and specific HG-VPF receptor binding are both valid mechanisms of HG-VPF-mediated calcium changes. Their potential relevance in the setting of microvascular permeability is discussed.

KEY WORDS • **glioma** • **edema** • **vascular permeability factor** • **dexamethasone** • **fluorescent calcium probe** • **endothelial cell** • **polycation**

VASOGENIC cerebral edema commonly accompanies malignant primary and metastatic brain tumors. It can also occur in certain benign tumors, such as meningioma. As the mass effect resulting from edema is often as culpable for neurological deterioration as the tumor mass, much interest has been focused on this troublesome clinical problem. Early investigations distinguished vasogenic cerebral edema on a morphological basis, from edema associated with cerebral ischemia, hydrocephalus, and water intoxication.[2,4,15] Studies of tumor vascular ultrastructure revealed variations from the typical morphology of blood-

brain barrier microvessels.[2,17–19] The finding of abundant micropinocytotic vesicles, capillary fenestrations, discontinuous tight junctions, and widened intercellular junctions made these tumor vessels appear inherently permeable to macromolecular substances. This premise is supported by radioisotope tracer studies which show the water component of vasogenic brain edema to be derived primarily from the tumor site, and not from the surrounding peritumoral cerebral vasculature. However, many cerebral neoplasms display similar morphological alterations in their microvasculature in the absence of cerebral edema. Furthermore, many features of tumor vessels are common to normal microvessels in extraneural tissues. Morphological changes alone do not, therefore, fully explain the occurrence of vascular extravasation.

Prior experience with a vascular permeability factor (VPF) derived from cultured human malignant glioma cells (HG-VPF)[3,5] prompted an inquiry as to whether this substance acted directly upon isolated endothelial cells. Considerable evidence exists linking well-characterized mediators of vascular permeability (histamine, bradykinin) with changes in endothelial cytosolic calcium, reduction of F-actin content, cytoskeletal alterations, and subsequent microvascular leakage.[6,16,21,22,24,25,28,30] The effect of HG-VPF upon this apparently relevant physiological cascade was investigated. To accomplish this, the fluorescent calcium ion probe fura-2/AM was used to determine whether HG-VPF could induce alterations in cytosolic calcium homeostasis in isolated endothelial cells *in vitro*. The endothelial cell cultures used were obtained from organs (placenta, lung, adrenal, brain) of several species (human, bovine, rat), and were representative of both macrovascular and microvascular, as well as arterial and venous systems.

## Materials and Methods

### *Human Glioma Cell Culture*

Cells derived from a cloned human malignant glioma (U251) were used as the source of HG-VPF activity. They were cultured at 37°C in 95% air and 5% $CO_2$, at 100% humidity in Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine (4 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% fetal calf serum (FCS). Newly confluent cultures grown in Falcon tissue culture flasks (175 sq cm growth area) were washed three times with Hanks' balanced salt solution (HBSS) without calcium, magnesium, or phenol red and incubated with 25 ml of serum-free DMEM for 24 hours; next, they were again rinsed and placed in 30 ml of fresh serum-free DMEM. The cells were then allowed to incubate for 5 to 7 days and the conditioned serum-free medium was harvested.

Flasks of confluent cells were subcultured, using standard trypsinization procedures, every 5 to 20 days as necessitated by biological growth patterns. Confluent flasks contained $6 \times 10^7$ to $8 \times 10^7$ cells with viability greater than 98% as determined by the trypan blue dye-exclusion method. Staining for glial fibrillary acidic protein (GFAP) confirmed the glial origin of the stock cultures. Stock cultures were periodically monitored for *Mycoplasma* infection.

### *Preparation of Human Glioma-Derived Protein*

Conditioned medium was stored frozen at −20°C, and later concentrated 20- to 40-fold in 3- to 6-liter batches, using wet-tubing dialysis* against 0.05 M ammonium bicarbonate, titrated to pH 7.4 with glacial acetic acid, followed by shell freezing in super-cooled acetone and lyophilization for 48 hours. Lowry protein determinations[20] were performed and proved 95% positive for lyophilized sample weight. The lyophilized product was reconstituted in Dulbecco's phosphate-buffered saline without calcium, magnesium, or phenol red, and filtered through a 0.45-μm membrane† before assaying for HG-VPF activity. Removal of VPF activity, as determined by the Miles assay, was achieved by heparin affinity chromatography.[5] This flow-through (non-VPF-containing) product served as a useful negative control for other brain tumor-derived substances present in the lyophilized product. However, limitations in the quantity of VPF activity available required the use of partially purified product (dialyzed/lyophilized, non-heparin-purified) as the VPF stimulus for the current studies. Previous determinations have shown HG-VPF to comprise less than 0.01% of this crude extract.[5]

### *Miles Microvascular Permeability Assay*

The Miles assay is a biological assay for the induction of capillary permeability in normal skin following the intradermal injection of test substances. A modification of the original assay, described previously for brain tumor-derived HG-VPF activity,[3,5] was used in this study. After induction of anesthesia with diethyl ether, previously shaved 450- to 500-gm male Hartley-strain guinea pigs‡ were given an intracardiac injection of 1% Evans blue dye and 10 μCi ($2.22 \times 10^7$ disintegrations per minute (dpm)) of $^{125}$I-bovine serum albumin ($^{125}$I-BSA, specific activity 2.0 μCi/μg)§ in a total volume of 1.0 ml, using a No. 25 19-mm long winged infusion set.‖ Five minutes later, 50 μl of individual test samples was injected intradermally via No. 30 needles affixed to 1.0-ml tuberculin syringes. All samples were tested in triplicate or quadruplicate. Histamine standards (0.5 to 1.0 μg/50 μl) were included when indicated.

Test samples that induced cutaneous microvascular

* Dialysis tubing, 25,000 MWCO, manufactured by Spectrum Medical Industries, Inc., Los Angeles, California.

† Dulbecco's phosphate-buffered saline supplied by GIBCO Laboratories, Grand Island, New York; membrane supplied by Gelman Sciences, Ann Arbor, Michigan.

‡ Guinea pigs obtained from Charles River Laboratories, Wilmington, Massachusetts.

§ $^{125}$I-BSA obtained from Dupont-New England Nuclear, Boston, Massachusetts.

‖ Infusion set manufactured by Terumo Corp. Tokyo, Japan.

permeability caused a visible blue stain at the injection site due to extravasation of the Evans blue dye-albumin complex. After 30 minutes, the animals were exsanguinated and the injection sites were excised with a 5-mm circular leather-punch. This method produces skin tags of constant surface area and weight, which consistently encompassed the small areas of dye extravasation produced by the 50-$\mu$l injection volumes. The VPF activity within these injection sites was quantified directly using a gamma counter and is expressed as dpm.

*Endothelial Cell Isolation and Culture*

Human umbilical vein endothelial cells were obtained by collagenase digestion of umbilical cords collected at Caesarean section,[9,13,14] or obtained directly from the American Type Culture Collection (ATCC).* Harvested cells were plated in flasks coated with 2% gelatin containing medium-199 supplemented with L-glutamine (2 mM), heparin (100 $\mu$m/ml), thymidine (10 $\mu$M), 15% FCS, 10% conditioned medium (derived from normal glia, glial tumor, and a variety of endothelial cell cultures), and endothelial cell growth supplement (50 $\mu$g/ml). All cell cultures were maintained at 37°C in 95% air and 5% $CO_2$ at 100% humidity. At confluence, cultures were suspended in trypsin-ethylenediaminetetra-acetic acid (EDTA, 0.05%) and passaged at a 1:4 split ratio. Stock cultures at passage 1 or 2 were routinely frozen in 5% dimethyl sulfoxide, 5% dextran-70, and 90% FCS by means of a programmable cell freezer;† they were then stored in vapor-phase nitrogen, and periodically thawed and replanted in medium-199 supplemented as above. Fura-2/AM studies were restricted to low-passage cultures that displayed morphological characteristics of vascular endothelium under phase-contrast microscopy. Furthermore, their endothelial identity was confirmed by Factor VIII immunofluorescent staining and uptake of acetylated low-density lipoprotein (LDL).[9,13,14,36] Contamination by cells with morphological features and the growth characteristics of smooth-muscle cells was not observed.

Bovine pulmonary artery endothelial cells‡ were obtained directly from ATCC at Passage 22. They had previously been characterized as having an intact glycocalyx and surface receptors, as well as having positive immunofluorescent staining for Factor VIII and angiotensin-converting enzyme.[26,27] In addition, they displayed typical cobblestone monolayer morphology when examined by phase-contrast microscopy. These cells were cultured at 37°C in 95% air and 5% $CO_2$ at 100% humidity in medium-199 containing Earle's salts and L-glutamine (4 mM), supplemented with heparin (100 $\mu$g/ml), thymidine (10 $\mu$M), sodium bicarbonate (4.35 gm/liter), penicillin (100 U/ml), and streptomycin (100 $\mu$g/ml). Cultures were grown on gelatin-coated flasks and subcultured at a split ratio of 1:2 upon reaching confluence.

Bovine adrenal medullary endothelial cells were obtained by collagenase digestion of adrenal glands obtained fresh from the abattoir. Isolation of pure endothelial cell lines was achieved by gross dissection of digested glands, passage of digested adrenal medullary tissue through a series of nylon mesh filters, and by differential plating. Their endothelial identity was confirmed by immunofluorescent staining for Factor VIII and uptake of acetylated LDL (DiI-Ac-LDL), and the absence of cells staining for smooth-muscle actin. Phase-contrast microscopy revealed a characteristic propensity of the cells to form capillary-like structures when grown in monolayers. Cultures were maintained at 37°C in 95% air and 5% $CO_2$ at 100% humidity in Eagle's modified essential medium supplemented with L-glutamine (2 mM), endothelial cell growth supplement (20 $\mu$g/ml), penicillin (100 U/ml), streptomycin (100 $\mu$g/ml), and 10% FCS. This cell line was routinely grown on uncoated flasks. Passage was carried out using trypsin-EDTA (0.05%) and subculturing at a 1:4 split ratio.

Cerebral microvessel endothelial cells were obtained by harvesting Fisher 344 rat brains. Gentle ether anesthesia was followed by rapid decapitation and aseptic removal of the cerebral tissue. The brains were then washed in 10:1 volumes HBSS and minced in media containing both antibiotics and antimycotics until a uniform slurry of tissue particles less than 1.0 mm was achieved. The slurry was then digested with protease (0.5% in 300 mM sucrose)§ for 30 minutes at 37°C, followed by neutralization of the enzymatic activity with 10% BSA. The tissue was homogenized with six to eight strokes using a loosely fitting glass pestle at slow speed. The final purification involved differential plating and clonal isolation of cells which showed typical morphological features on phase-contrast microscopy, and fluorescent staining for Factor VIII and uptake of DiI-Ac-LDL. The cells of interest showed no evidence of staining for GFAP or smooth-muscle actin. Cell cultures were maintained at 37°C in 95% air and 5% $CO_2$ at 100% humidity. The culture medium used consisted of medium-199 with L-glutamine (2.5 mM), Earle's salts, and a combined sodium bicarbonate/HEPES buffer system (2.0 gm/liter and 10 mM). This medium was further supplemented with endothelial cell growth supplement (25 $\mu$g/ml), heparin (100 $\mu$g/ml), thymidine (10 $\mu$M), 12.5% FCS, 15% U251 glioblastoma clone conditioned medium (48 to 96 hours old), 5% to 10% rat brain glial and endothelial cell conditioned medium, penicillin (100 U/ml), streptomycin (100 $\mu$g/ml), and amphotericin B (2.5 $\mu$g/ml for the first week only).

* Human umbilical vein endothelial cells obtained from the American Type Culture Collection, Rockville, Maryland.

† Programmable cell freezer manufactured by Cryo-Med, Inc., Mount Clemens, Michigan.

‡ Bovine pulmonary artery endothelial cells supplied by the American Type Culture Collection, Rockville, Maryland.

§ Dispase type II sucrose obtained from Boehringer Mannheim, West Germany.

*Fura-2/AM Loading*

Confluent cultures were washed with $Ca^{++}/Mg^{++}$-free HBSS and incubated at 37°C for 5 to 10 minutes in the same buffer containing 25 mM EDTA. This method minimized alteration of surface receptors and membrane proteins that might otherwise be damaged by standard trypsinization techniques. Retracted cells were readily suspended by gentle agitation, diluted in an equal volume of HBSS, pelleted, washed, and resuspended in HBSS. Endothelial cells (approximately $10^7$ cells/ml medium) were routinely loaded by incubation with 5.0 $\mu$M fura-2/AM for 30 minutes. A loading incubation temperature of 15° to 20°C was used to minimize nonhomogeneous concentration of the probe by sequestration into perinuclear acidic organelles.[23] The residual extracellular fura-2/AM was then diluted 1:10 by adding the appropriate quantity of medium, and the cells were again allowed to equilibrate at 15° to 20°C for 30 minutes. The cells were gently washed three times by pelleting (8000 rpm × 5 minutes) and then resuspending them in 20 to 30 ml of culture medium not containing fura-2/AM. The final pellet was suspended in 1 ml of medium and contained $2 \times 10^7$ cells/ml. This suspension was stored on ice until used. Preliminary experiments showed negligible fura-2 leakage (< 5% after storage for up to 4 hours) and experiments were routinely completed within that interval.

*Fluorescence Measurements*

Fluorescence measurements were performed using a spectrofluorometer equipped with a temperature-controlled magnetically stirred cuvette holder and interfaced with a spectroscopy laboratory coordinator and a recorder or printer.‖ All assays were conducted at 37°C. Excitation and emission wavelengths were 340 and 510 nm, respectively; the gain was adjusted to provide a fluorescence of approximately 50% of full scale. The data collected were real-time recordings after establishing a baseline steady state for at least 2 minutes. Assays were conducted in disposable plastic cuvettes loaded with $2 \times 10^6$ cells (final concentration approximately $1 \times 10^6$ cells/ml) suspended in 2 ml of modified Krebs-Ringer solution containing 35 mM NaCl, 5 Mm KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES and adjusted to a pH of 7.4 ± 0.1 with NaOH. Calibration of fura-2 fluorescence was performed as previously described.[24] Maximum and minimum fluorescence values were recorded at $10^{-3}$ M $Ca^{++}$ and $10^{-9}$ M $Ca^{++}$ by first lysing cells in Triton X-100 or digitonin, respectively, followed by chelation of free $Ca^{++}$ on addition of 4 mM ethyleneglycol-bis ($\beta$-aminoethylether)-N,N'-tetra-acetic acid (EGTA) and sufficient Tris base to bring the pH to above 8.3. All test substances, inhibitors, and calibrating reagents were added as 5 to 50 $\mu$l aliquots (0.25% to 4.0% of the sample cuvette volume). Fluorescence values were not corrected for the minimal resulting sample dilution.

‖ Fluorolog spectrophotometer and Model DM1B spectroscopy laboratory coordinator manufactured by SPEX Industries, Inc., Edison, New Jersey; R-100A recorder manufactured by Perkin-Elmer Corp., Norwalk, Connecticut; HIPLOT printer, Model DMP-40, manufactured by Bausch and Lomb, Austin, Texas.

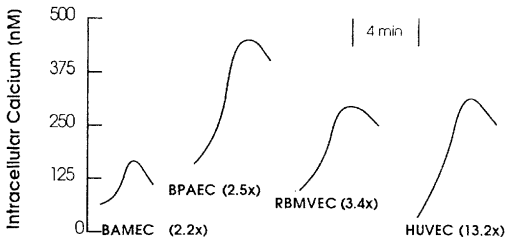


FIG. 1. Induction of cytosolic calcium transients in various endothelial cell lines by partially purified human glial tumor-derived vascular permeability factor (HG-VPF). The relative degree of intracellular calcium change induced by a standard HG-VPF stimulus is indicated in parentheses. All graphic data were obtained by computerized tracing of original hard-copy data after reassignment of y-axis values by conversion to absolute $Ca^{++}$ concentrations. BAMEC = bovine adrenal medullary endothelial cells; BPAEC = bovine pulmonary artery endothelial cells; RBMVEC = rat brain microvessel endothelial cells; HUVEC = human umbilical vein endothelial cells.

Cytosolic calcium concentrations $[Ca^{++}]_i$ were calculated, based on a dissociation constant (Kd) of 224 nM for $Ca^{++}$ bound to fura-2, by the following equation:

$$[Ca^{++}]_i = \frac{F_{observed} - F_{minimum}}{F_{maximum} - F_{observed}} \times Kd_{fura-2-Ca^{++}}.$$

Figures 1 through 6 represent computer-generated tracings of the original hard-copy real-time data. This was accomplished after conversion of the y-axis peak emission values, using the formula shown above to arrive at absolute intracellular calcium values $[Ca^{++}]_i$ expressed in nanomolar concentrations (nM).

## Results

The basal level of $[Ca^{++}]_i$ in unstimulated endothelial cells was found to be between 50 and 100 $\mu$M, irrespective of the tissue source of the cells. Human glioma-derived VPF induced significant cytosolic calcium ion transients in all endothelial cell lines examined (Fig. 1); however, it did not elicit a calcium response in two non-endothelial cell lines, U251 glioma and fibroblasts (data not shown). Endothelial responses were typically rapid or slightly delayed in onset (15 to 45 seconds), and varied in magnitude depending upon the cell type being studied. The largest responses were observed in human endothelial cells. Peak HG-VPF-induced $[Ca^{++}]_i$ elevations were attained within 60 seconds, exhibited a dose-response phenomenon, and were followed by a sustained elevation above baseline for 5 to 10 minutes thereafter (Fig. 2). In contrast, exposure of endothelial cells to the flow-through product of human glioma-derived conditioned medium after binding of all HG-VPF activity to a heparin-Sepharose affinity

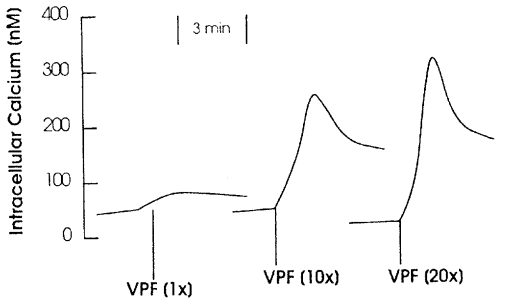


FIG. 2. Dose-response relationship of cytosolic calcium transients induced by human glioma-derived vascular permeability factor (VPF) in human umbilical vein endothelial cells. Fold concentration is expressed as multiples of a standard solution containing 0.25 mg/ml (1x) of partially purified lyophilized glioma-conditioned medium dissolved in Dulbecco's phosphate-buffered saline (2.5 mg/ml = 10x, 5.0 mg/ml = 20x). See Fig. 1 for origin of data.

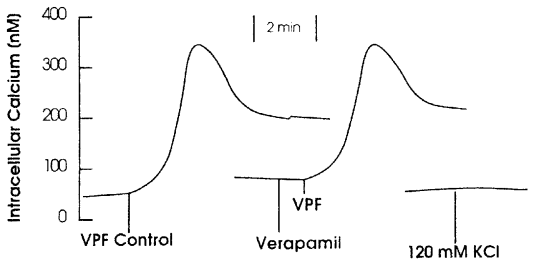


FIG. 3. Failure of verapamil to inhibit human glioma-derived vascular permeability factor (VPF)-induced calcium transients. Prior exposure of endothelial cells to $10^{-6}$ M verapamil did not diminish cytosolic calcium changes induced by VPF. Calcium transients were not induced by exposure of endothelial cells to 120 mM KCl. This suggests that VPF-induced influx of $[Ca^{++}]_e$ occurs by non-voltage-gated calcium ion channels. See Fig. 1 for origin of data.

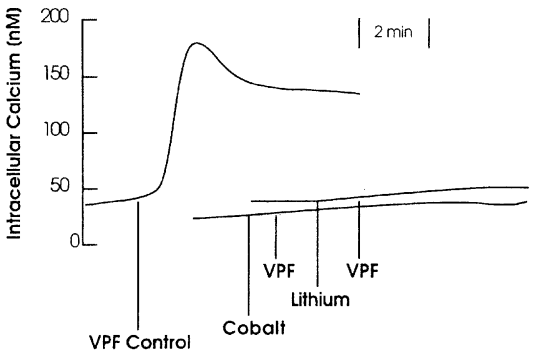


FIG. 4. Inhibition of human glioma-derived vascular permeability factor (VPF)-induced calcium ion transients by 2 mM $Li^+$ and $Co^{++}$ cations. Similar results were obtained with 2 mM concentrations of $Mn^{++}$ and $La^{++}$ cations (data not shown). Nonspecific cationic calcium channel blockers appear to inhibit VPF-induced intracellular calcium transients. This suggests that influx of extracellular calcium via non-voltage-gated membranous channels may be the primary event in the VPF-elicited response. See Fig. 1 for origin of data.

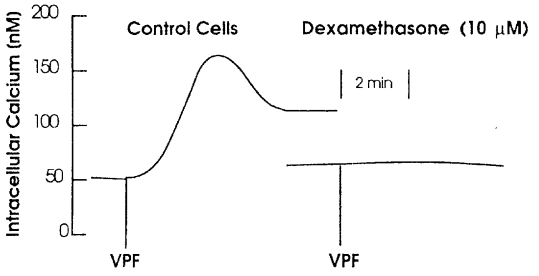


FIG. 5. Inhibition of human glioma-derived vascular permeability factor (VPF)-induced cytosolic calcium ion transients in endothelial cells incubated 2 hours with 10 $\mu$M dexamethasone. See Fig. 1 for origin of data.

column* did not produce a change in intracellular calcium. This flow-through product has previously been shown to largely contain tumor-derived proteins exclusive of HG-VPF, as determined by an *in vivo* assay of microvascular permeability.[5] The brief time course of the HG-VPF-induced calcium changes are compatible with other data showing prolonged stimulus-response coupling to occur after only transient intracellular calcium elevations.[25]

As elevated extracellular potassium chloride (120 mM $[K^+]_e$) did not induce cytosolic calcium transients, it is unlikely that HG-VPF acts via voltage-gated calcium channels. Similarly, verapamil ($10^{-6}$ M) failed to inhibit HG-VPF-induced calcium changes. This suggests that HG-VPF increases cytosolic $Ca^{++}$ by a mechanism other than calcium entry via verapamil-sensitive calcium channels (Fig. 3). Furthermore, addition of 2 mM $Li^+$, $Co^{++}$, $Mn^{++}$, or $La^{+++}$ inhibited the HG-VPF-induced calcium transient *in vitro*, as did the absence of extracellular $Ca^{++}$ after chelation with EGTA. This indicates that HG-VPF-induced changes largely depend upon the influx of extracellular calcium through membranous calcium channels independent of the voltage-sensitive variety (Fig. 4). Finally, incubation of endothelial cells for 2 hours with 10 $\mu$M dexamethasone before exposure of HG-VPF-containing medium resulted in inhibition of the HG-VPF-induced cytosolic changes (Fig. 5). This inhibitory effect was not observed in similarly treated cells exposed to cyclic adenosine monophosphate, suggesting that the steroid-induced inhibition was specific for HG-VPF-induced calcium influx.

Another polycationic substance (protamine) induced changes in intracellular calcium (Fig. 6 *left*) at concentrations as low as 10 $\mu$g/ml. Induction of calcium transients by protamine was rapid in onset, peaked within 30 seconds, and sustained a level above baseline for a number of minutes thereafter. The presence of competing extracellular divalent cations (2 mM $Co^{++}$ and $Mn^{++}$) reduced peak $[Ca^{++}]_i$, but more completely affected the post-maximum levels, resulting in a rapid

* Column obtained from Pharmacia, Inc., Piscataway, New Jersey.

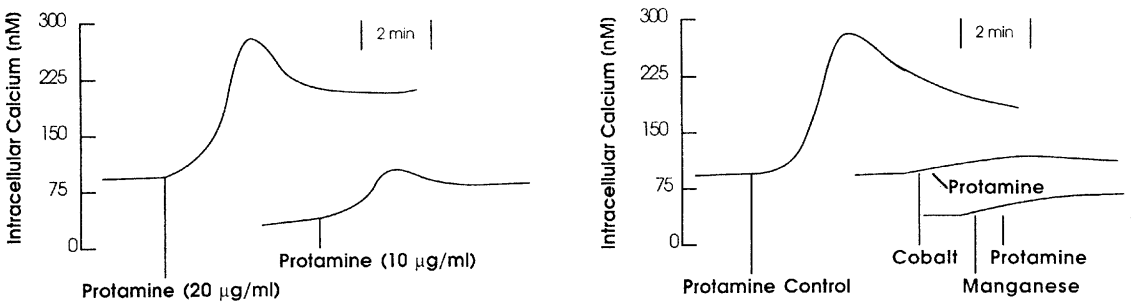


FIG. 6. *Left:* Induction of cytosolic calcium ion transients by protamine. *Right:* Inhibition of polycation-induced calcium ion flux in endothelial cells by the nonspecific calcium channel blockers $Co^{++}$ and $Mn^{++}$.

downslope and normalization of intracellular calcium (Fig. 6 *right*). These findings indicated that combined mobilization of intracellular calcium stores (rapid upslope and peak levels) as well as influx from the extracellular compartment (peak and sustained post-maximum levels) are involved in the response to HG-VPF and protamine. Moreover, the effects of HG-VPF and protamine were inhibited in the presence of heparin, a polyanionic glycoprotein (data not shown).

## Discussion

Cytosolic free $Ca^{++}$ plays important roles in many physiological cellular processes including reduction of F-actin content, cytoskeletal alterations, and subsequent microvascular extravasation.[6,10,12,16,22–25,28,30,32,33] Studies of cytosolic calcium changes have recently been facilitated by novel calcium ion chelators (quin-2/AM, indo-1/AM, and fura-2/AM) possessing molecular structures similar to EGTA.[11,33] They are available as sodium salts and acetoxy-methyl esters. The latter form is useful for intracellular $Ca^{++}$ determinations. These agents differ from EGTA in that they possess aromatic rings capable of electrostatic interactions with the functional groups that participate in the chelation of $Ca^{++}$. This interaction during chelation changes the fluorescence and ultraviolet light-absorption properties of these molecules. The spectral changes allow these molecules to be used as sensitive probes for divalent cations.

Fura-2/AM is particularly useful in that it is highly specific for $Ca^{++}$ ions, readily distinguishing them from other divalent cations. Furthermore, it is sensitive to $Ca^{++}$ changes in the nanomolar range that are encountered within endothelial cells. Fura-2 produces a potent fluorescent emission ($30 \times F_{quin\text{-}2}$) which allows its use in relatively low concentrations, thereby lowering any interference due to an inherent $Ca^{++}$ buffering capacity. Because of its lipophilicity, fura-2/AM is rapidly internalized by endothelial cells. Once internalized, deesterification of the acetoxy-methyl group (fura-2/AM—>fura-2) converts it to the free acid (lipophobic form), which has minimal capability to diffuse out of the cell. Residual extracellular fura-2/AM, removed by a series of cell washings, assures that observed fluorescence changes correspond solely to alterations in intracellular $Ca^{++}$.

Human glioma-derived VPF is an acid-stable, heat labile protein of 41- to 56-kD molecular weight.[3,5] Coincubating glial tumor cells with concentrations of dexamethasone compatible with those achieved in patients being treated for neoplastic brain edema inhibits expression of HG-VPF.[5] Activity is also inhibited by pretreating test animals with dexamethasone, an effect which is partially reversed by exposing the test animals to inhibitors of protein synthesis before dexamethasone treatment.[5] Inhibition of HG-VPF activity by dexamethasone *in vitro* and *in vivo* correlates well with the efficacy of this agent in treating neoplastic vasogenic cerebral edema. It is possible that HG-VPF may act upon the prostaglandin cascade via activation of phospholipase-$A_2$, the rate-limiting enzyme in that pathway. This postulate is based upon evidence of inhibition of VPF activity by antagonists of cyclo-oxygenase (indomethacin) and by dexamethasone.[3,5] The latter agent potently inhibits phospholipase-$A_2$ indirectly by inducing synthesis of an intermediate referred to as "lipocortin," "macrocortin," or "endocortin."[1,7,8] The effects of dexamethasone upon VPF activity have been shown to be inhibited by prior exposure to protein synthesis inhibitors,[5] thus suggesting a link to this steroid-inducible cascade, and therefore phospholipase-$A_2$ (and prostaglandins). Other investigators have shown prostaglandins to be potent inducers of calcium transients in endothelial cells.[8] The observation that dexamethasone inhibition of HG-VPF occurs through a direct interaction with the endothelial cell provides new insight into the use of corticosteroids, which has hitherto been largely empirical in this setting.

Human glioma-derived VPF is a positively charged (cationic) protein which has an affinity for heparin,[3,5,29] a polyanionic substance coating up to 85% of the endothelial cell surface. Exposure of endothelial cell suspensions to heparin before administration of HG-VPF inhibits the induction of calcium transients by HG-VPF and protamine. The mechanism by which surface charge neutralization by polycationic molecules (HG-VPF and protamine) results in alterations in intracellular calcium is not known. In fact, the data available

at this time do not eliminate the possibility of a specific HG-VPF cell-surface receptor interaction. A number of studies have demonstrated and discussed the induction of microvascular permeability by polycationic substances. Intracarotid infusion of protamine produces vascular extravasation of Evans blue dye into the rabbit brain.[31] Similarly, poly-1-lysine, another positively charged molecule, produces protein leakage (nephrotic syndrome) after exposure of the renal vasculature in rats.[34,35] It has been hypothesized that these cationic agents act by neutralizing the negatively charged (anionic) endothelial cell surface. Ultrastructural studies have shown a reorganization of renal epithelial podocyte processes in the poly-1-lysine-induced nephrotic syndrome.[35] However, there is no prior direct evidence to support and explain the physiological basis of these interactions. It was found that, like VPF, protamine produces significant cytosolic calcium transients in fura-2-loaded endothelial cell suspensions. Furthermore, this action is attenuated by the presence of extracellular divalent cations capable of competing with calcium for transmembranous ion channels. Premixing protamine with heparin also inhibited the action of protamine upon the endothelial cells.

Further investigation of the subcellular mechanisms of HG-VPF activity is warranted. This will require increasingly pure preparations of this substance, as well as antibodies directed against its active site and putative receptor. Increased understanding of the mechanism of neoplastic cerebral edema may lead to improved treatments for this important accompaniment of intracranial tumors. Possibilities suggested by the results of experiments reported here include the use of specific calcium channel blocking agents or less specific cationic salts (lithium carbonate) to treat vasogenic cerebral edema associated with brain tumors. These substances may ultimately prove to be useful adjuncts, if not alternatives, to high-dose corticosteroid therapy.

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*Address reprint requests to:* Gregory R. Criscuolo, M.D., Attn. Mrs. Lynn Schofield, National Institutes of Health, Surgical Neurology Branch — NINDS, 9000 Rockville Pike, Building 10, Room 5D-37, Bethesda, Maryland 20892.