Short-term Regulation of Na^+/K^+ Adenosine Triphosphatase by Recombinant Human Serotonin 5-HT_{1A} Receptor Expressed in HeLa Cells

John P. Middleton, John R. Raymond, A. Richard Whorton, and Vincent W. Dennis
Duke University Medical Center, Department of Medicine, Durham, North Carolina 27710

Abstract

Agonist occupancy of the cloned human serotonin (5-HT)$_{1A}$ receptor expressed in HeLa cells stimulates Na^+/K^+ ATPase activity as assessed by rubidium uptake. The purpose of the study was to determine which of the receptor-associated signaling mechanisms was responsible for this effect. 5-HT stimulated Na^+/K^+ ATPase 38% at 2 mM extracellular potassium, an effect characterized by a decrease in apparent $K_{0.5}$ from 2.8±0.3 to 1.8±0.3 mM potassium without a significant change in apparent V_{max}. The EC_{50} for the transport effect was ~ 3 μM 5-HT. The response was pertussis toxin-sensitive but did not involve inhibition of adenylate cyclase, as stimulation of Na^+/K^+ ATPase by 5-HT was observed in the presence of excess dibutyryl cAMP. Protein kinase C was not required for the response since short-term incubation with the phorbol esters phorbol 12 myristate, 13 acetate (PMA) and phorbol 12,13-dibutyrate (PDBu) did not mimic the 5-HT effect. Moreover, 5-HT increased Na^+/K^+ ATPase activity after inactivation of protein kinase C by overnight incubation with PMA. 5-HT and the sesquiterpene lactone thapsigargin increased cytosolic calcium in this cell model, and the EC_{50} for 5-HT corresponded with that for stimulation of Na^+/K^+ ATPase. Both thapsigargin and A23187, a calcium ionophore, also increased Na^+/K^+ ATPase activity in a dose-responsive fashion. The response to 5-HT, thapsigargin, and A23187 was blocked by conditions that removed the cytosolic calcium response. By two-dimensional gel electrophoresis, we established evidence for a calcium-sensitive but protein kinase C-independent signaling pathway. We conclude that the 5-HT_{1A} receptor, which we have previously shown to stimulate phosphate uptake via protein kinase C, stimulates Na^+/K^+ ATPase via a calcium-dependent mechanism. This provides evidence for regulation of two separate transport processes by a single receptor subtype via different signaling mechanisms. (*J. Clin. Invest.* 1990. 86:1799–1805.) Key words: protein kinases • serotonin • Na^+/K^+ ATPase • cytosolic calcium • adrenergic receptor

Introduction

At least seven subtypes of serotonin (5-HT)[1] receptors are linked to various signal transduction pathways and biologic effects (1). The human 5-HT_{1A} receptor has been isolated, cloned, and expressed in HeLa cells, a cell line derived from human cervical epithelium, where it displays three distinct effects on signaling pathways: inhibition of adenylate cyclase, activation of protein kinase C, and increased intracellular calcium concentration (2–4). Although much is known about 5-HT receptors and their associated signaling pathways, relatively little is known about their biologic effects.

We recently showed that the human 5-HT_{1A} receptor expressed in HeLa cells increases sodium-dependent phosphate transport via activation of protein kinase C (2). One of the potential mechanisms for this effect on phosphate uptake would be increased primary active transport of sodium via Na^+/K^+ ATPase. Though the present studies confirm this possibility, they importantly demonstrate that 5-HT increases Na^+/K^+ ATPase activity via a branch of the phospholipase C pathway different from that which increases sodium-dependent phosphate uptake. Specifically, increased cytosolic calcium, and not necessarily activation of protein kinase C, is required for the response. These studies therefore demonstrate that a single receptor subtype may affect different but related transport events via simultaneous activation of divergent signaling pathways.

Methods

Cell culture. Constitutive gene expression was achieved in HeLa cells using the procedure detailed elsewhere (2, 4). As previously documented, the level of expression in the transfected HA-6 cell line is 2.8±0.8 pmol of 5-HT_{1A} receptor/mg protein (2). The level of receptor expression was determined every four to five passages using radioligand ([^{3}H]-8-OH-DPAT) binding.

HeLa cells, both "wild-type" and transfected clones (designated as HA-6 cells), were grown in monolayers in DMEM supplemented with 10% FCS, penicillin (100 U/ml) and streptomycin (100 μg/ml). Unless noted otherwise, cells were seeded in plastic culture wells or dishes (Costar Nuclepore Corp., Cambridge, MA) and incubated at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. All culture reagents were obtained from Gibco Laboratories (Grand Island, NY). Medium was changed to serum-free DMEM with 0.1% BSA 6–8 h before experimentation. For pertussis toxin (PTx) (List Biological Laboratories, Inc., Campbell, CA) experiments, 100 ng/ml PTx was added to serum-free medium 4 h before study.

Transport studies. Previous studies have validated the substitution of rubidium transport for that of potassium as an estimate of Na^+/K^+ ATPase in HeLa cells (6, 7). To improve access to the Na^+/K^+ ATPase, subconfluent monolayers were grown on collagen-coated filters on supports (Costar). After incubation in Earle's solution (Na^+ 143 mM, K^+ 5.4 mM, Mg^{2+} 0.8 mM, Ca^{2+} 1.8 mM, Cl^- 125 mM, Hepes 15 mM, PO_4^{2-} 0.2 mM, pH 7.4) with or without agonist, filters were washed in medium and immersed for 4 min at 22°C in phosphate-free Earle's solution that contained a specified potassium concentration and ^{86}Rb (≈ 4,000 cpm/nmol K^+) with or without 1 mM ouabain. In these conditions, rubidium uptake was linear through this time point (not shown). Filters were washed with 145 mM sodium chloride at 0°C and solubilized in scintillation fluid for counting. When monolayers were studied in similar conditions on conventional culture dishes only 20–30% of rubidium uptake was ouabain-sensitive compared with 85–95% in monolayers cultured on filters. For calcium depletion stud-

Address reprint requests to Dr. John Middleton, Box 3014, Duke University Medical Center, Durham, NC 27710.

Received for publication 12 December 1989 and in revised form 6 July 1990.

1. *Abbreviations used in this paper:* 5-HT, serotonin; G, guanine; IBMX, isobutyl-methylxanthine; IP_3, inositol 1,4,5-trisphosphate; MARCKS, myristoylated alanine-rich C-kinase substrate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PMA, phorbol 12 myristate, 13 acetate; PTx, pertussis toxin.

J. Clin. Invest.

0021-9738/90/12/1799/07 $2.00
Volume 86, December 1990, 1799–1805

ies, cell monolayers were incubated in Ca^{2+}-free Earle's medium with 1 mM EGTA for 15 min before agonist stimulation.

Intact cell phosphorylation. Phosphorylation of proteins in intact cells was done as described previously (2, 8, 9). Phosphorylation of the myristoylated alanine-rich C-kinase substrate (MARCKS) protein was used as an indicator of protein kinase C activity (2, 8, 9). A physiologic buffer was used to incubate the monolayer with $^{32}PO_4$ (100 μCi/ml) for 2 h before treatment with agonist. The monolayer was washed, cells scraped and suspended in 50 mM Tris/HCl, 1 mM EGTA, 1.5 mM $MgCl_2$, 10 mM benzamidine, 100 mM NaF, 5 mM dithiothreitol, 0.25 M sucrose, and 2% Triton X-100, pH 7.4, at 0°C. After centrifugation at 50,000 *g* for 20 min, the supernatant proteins were precipitated with 20% TCA, solubilized in a lysis buffer of 9 M urea, 1% Nonidet, 2.5% 2-mercaptoethanol, and 1% ampholines, and prepared for two-dimensional gel electrophoresis.

Protein kinase C inactivation was achieved by overnight incubation in serum-free medium with 16 mM phorbol 12,13 β myristic acid (PMA), a method we have previously shown to decrease phorbol ester-induced MARCKS protein phosphorylation in this cell model (2).

Cytosolic calcium. Cells were grown to confluence on glass coverslips and loaded for 1 h at 37°C with 20 μM Fura 2 AM (Calbiochem Corp., San Diego, CA) in HBSS containing 0.01% albumin and 10 mM Hepes at pH 7.4. Coverslips were placed into a cuvette and continuously superfused at 37°C with HBSS. The cuvette was placed into a heated cuvette holder of a spectrofluorimeter (SFM 25; Kontron Analytical, Kontron Electronics Inc., Redwood City, CA), and the emission was monitored at 510 nm over the excitation spectrum from 310–410 nm. Excitation wavelengths were alternated by a microcomputer (5–6 s/cycle) and the emission data ratio (350/380 nm excitation) was calculated. Cytosolic Ca^{2+} concentration was calculated as described by Grynkiewicz et al. (10).

Statistical methods. Kinetics were evaluated by the Enzfitter nonlinear regression program (Elsevier-BIOSOFT; Cambridge, UK), and simple weighting was used to derive the kinetic values. Results were compared with paired *t* test as appropriate.

Results

Fig. 1 shows that 5-HT increased rubidium uptake in HA-6 cells, HeLa cells with stable expression of the 5-HT_{1A} receptor. This stimulation was receptor-mediated as supported by three findings: first, 10 μM 5-HT increased rubidium uptake 38% in transfected (HA-6) cells but not in nontransfected HeLa cells; second, in HA-6 cells the effect was blocked by methiothepin, a 5-HT_{1A} receptor antagonist (11); and third, the response was mimicked by 8-OH DPAT, a specific 5-HT_{1A} agonist (11, 12). The time course of the transport response indicated maximal 5-HT effect at 5–10 min, with return of rubidium uptake to basal rates after 30 min.

Fig. 2 *A* shows the effects of varying concentrations of potassium on rubidium uptake by transfected HA-6 cells. Rubidium uptake was a saturable process which was > 85% ouabain-sensitive. At 1.0, 2.0, and 5.4 mM potassium, ouabain-insensitive uptakes were 1.9±0.6, 1.6±0.8, and 8.9±1.9 nmol/mg protein per 4 min, respectively, and were unchanged by 5-HT. 5-HT increased rubidium uptake 47.3±22.8% at 1 mM K ($P < 0.05$), 25.9±5.5% at 2 mM K ($P < 0.01$), and 12.2±5.1% at 5.4 mM K ($P < 0.05$; *n*, 6–9). A Hill plot of the ouabain-sensitive component of rubidium uptake (Fig. 2 *B*) shows the complex nature of the 5-HT effect on transport kinetics. First, 5-HT decreased apparent $K_{0.5}$ from 2.7±0.3 to 1.8±0.3 mM potassium ($P < 0.05$). Second, 5-HT had no demonstrable effect on either the Hill coefficient (1.1±0.1 to 1.2±0.2) or the apparent V_{max} (50.6±3.2 to 53.4±4.0 nmol/mg protein per 4 min). By analogy to enzyme kinetics, the decrease in apparent $K_{0.5}$ following 5-HT treatment suggests that 5-HT_{1A} receptor occupancy alters the affinity of the pump for extracellular potassium (13). All subsequent studies used 2 mM extracellular potassium, a concentration that approximated the apparent $K_{0.5}$ for the transporter and limited the

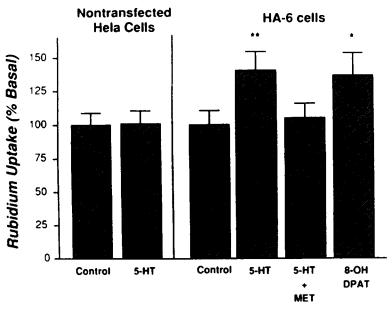

Figure 1. Effect of agonists on rubidium uptake in transfected and nontransfected HeLa cells. Cells were incubated for 5 min with 10 μM 5-HT in the presence or absence of 10 μM methiothepin (*MET*), or with 10 μM 8-OH DPAT. The results shown are for 5–9 separate experiments performed in duplicate. Control values were 25.4±5.2 nmol/mg protein per 4 min in nontransfected cells and 24.2±2.4 nmol/mg protein per 4 min for transfected cells at 2 mM extracellular potassium. (**$P < 0.01$; *$P < 0.05$).

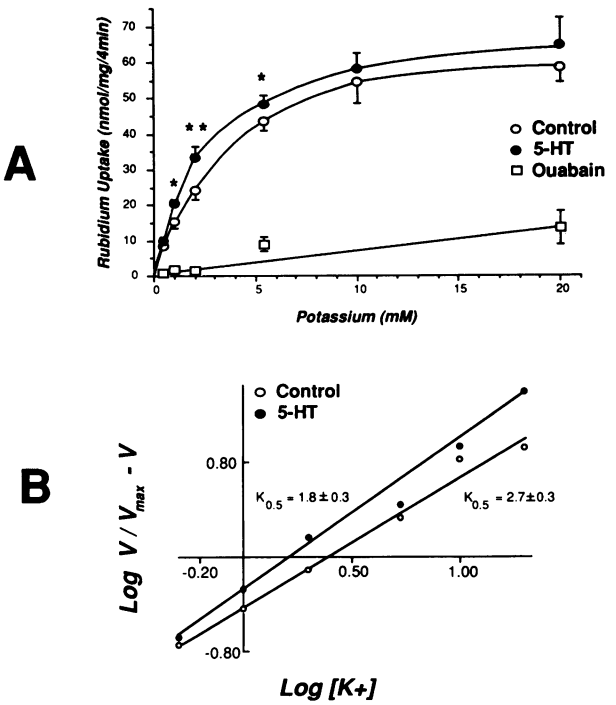

Figure 2. Kinetics of rubidium uptake in HA-6 cells. (*A*) Rubidium uptake was measured in Earle's solution with varying potassium concentration, with (*closed circles*) or without (*open circles*) 5 min incubation with 10 μM 5-HT. Uptake values in the presence of 1 mM ouabain are also shown (*squares*) and were not different for control or treated cells. Results represent means±SE from 6–9 experiments. (*B*) Hill plot of the ouabain-sensitive values from *A*. For untreated cells, the apparent $K_{0.5}$ was 2.7±0.3 mM potassium and the V_{max} was 50.6±3.2 nmol/mg protein per 4 min. For cells treated with 5-HT, the apparent $K_{0.5}$ was 1.8±0.3 mM potassium and the V_{max} was 53.4±4.0 nmol/mg protein per 4 min. The Hill coefficients, indicated by the slopes of the lines, were 1.1±0.1 for control and 1.2±0.2 for treated cells. (**$P < 0.01$; *$P < 0.05$).

ouabain-insensitive component of rubidium uptake to 6% of control values.

Because of the possibility that increased Na^+/K^+ ATPase activity observed with 5-HT might be the result of increased sodium entry, we compared the effect of 5-HT to that of nystatin, a polyene antibiotic that permeabilizes cells to sodium (14). Treatment of HeLa cells for 5 min with nystatin (160 μg/ml) had no demonstrable effect on rubidium uptake at 1 mM (23.6±1.2 to 22.6±0.5 nmol/mg protein per 4 min) or 2 mM (24.5±4.6 to 28.4±2.6 nmol/mg protein per 4 min) extracellular potassium concentration, conditions at which the 5-HT response was maximal. In contrast, nystatin increased rubidium uptake from 58.1±2.3 to 92.7±5.0 nmol/mg protein per 4 min at 10 mM potassium, and from 65.4±4.4 to 152.1±12.6 nmol/mg protein per 4 min at 20 mM potassium ($P < 0.01$; $n = 3$), conditions in which 5-HT showed no significant effect. This corresponded to a 2.5-fold increase in apparent V_{max} and a fourfold increase in the $K_{0.5}$ for potassium. In addition, at 2 mM potassium 5-HT increased rubidium uptake 13.4±3.0% ($P < 0.01$; $n = 6$) in the presence of 160 μg/ml nystatin. These two features suggest that the effect of 5-HT is not mediated by an increase in intracellular sodium: (*a*) stimulation of Na^+/K^+ ATPase by nystatin has distinctly different kinetic properties than that for 5-HT, and (*b*) the effect of 5-HT is retained in the presence of nystatin.

Fig. 3 shows the dose-response relationship between 5-HT and stimulation of rubidium uptake. The observed EC_{50}, ~ 3 μM 5-HT, corresponds with that described below for 5-HT stimulation of cytosolic calcium.

The guanine nucleotide binding (G) protein(s) linking the 5-HT_{1A} receptor in HA-6 cells with both inhibition of adenylate cyclase and activation of phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis are known to be pertussis toxin substrates (2, 4). Incubation of monolayers for 4 h with 100 ng/ml PTx prevented the effect of 5-HT on rubidium uptake (control, 22.6±1.8; 10 μM 5-HT, 24.0±2.0 nmol/mg protein per 4 min; $n = 7$). This information suggests the involvement of a PTx-sensitive G protein in the cellular signal, but it does not delineate the specific cell signal.

Fig. 4 addresses the possible role of receptor-mediated inhibition of adenylate cyclase in the action of 5-HT on Na^+/K^+ ATPase. Protein kinase A activation in HA-6 cells with 10 μM dibutyryl cAMP in the presence of 0.5 mM isobutylmethylxanthine (IBMX) decreased apparent V_{max} by 14.8±3.4% ($P < 0.05$; $n = 4$) without significantly altering either the $K_{0.5}$ or the Hill coefficient. Fig. 4 shows that the addition of 10 μM 5-HT in the presence of dibutyryl cAMP increased rubidium uptake to values similar to those observed in untreated cells.

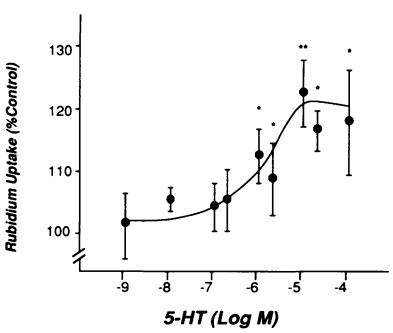

Figure 3. Dose-response relationship between 5-HT and rubidium uptake in HA-6 cells. Rubidium uptake was measured after incubation for 5 min with the given concentrations of 5-HT. Results represent means±SE from five to nine paired experiments. *$P < 0.05$; **$P < 0.01$.

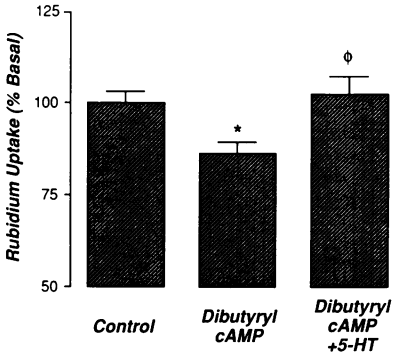

Figure 4. 5-HT stimulation of rubidium uptake in the presence of dibutyryl cAMP. Rubidium uptake was measured after incubation for 5 min with 0.5 mM IBMX and 10 μM dibutyryl cAMP with or without 10 μM 5-HT. Values are means±SE for 5–11 paired experiments. *$P < 0.005$ compared with basal; $\Phi P < 0.05$ compared with dibutyryl cAMP + IBMX alone.

These data demonstrate that the stimulation of Na^+/K^+ ATPase induced by 5-HT is not mediated by inhibition of adenylate cyclase.

We showed previously that 5-HT and phorbol esters activate protein kinase C in intact HA-6 cells, demonstrated by increased phosphorylation of the MARCKS protein, and that this activation mediates the effects of 5-HT on phosphate transport (2). As shown in Fig. 5, varying doses of active phorbols PMA and phorbol 12,13-dibutyrate (PDBu) had no demonstrable effects on rubidium uptake. In addition, 10 μM 5-HT increased rubidium uptake from 25.0±2.2 to 31.1±3.0 nmol/mg protein per 4 min ($P < 0.01$; $n = 7$) even after inactivation of protein kinase C by overnight treatment with PMA. These data indicate that the effect of 5-HT on rubidium uptake is not mediated by protein kinase C.

In addition to activation of protein kinase C, 5-HT increases intracellular calcium in HA-6 cells. The initial cytosolic calcium response is presumably initiated through inositol 1,4,5-trisphosphate (IP_3), which increases two- to threefold after 1.5–15 min of incubation with 10 μM 5-HT (2, 4). Fig. 6 shows the changes in intracellular calcium concentration in response to either 5-HT or thapsigargin. Thapsigargin is a ses-

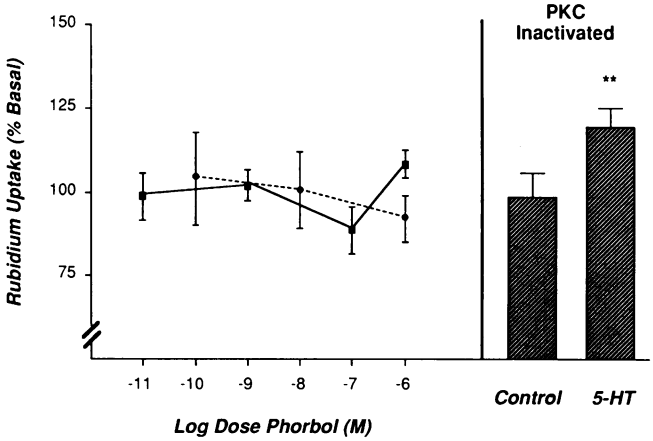

Figure 5. Effects of protein kinase C activity on rubidium uptake. On the left, rubidium uptake results are shown after exposure to either PMA (*solid line*) or PDBU (*dashed line*) for 5 min. On the right, results are shown for HA-6 cells with inactivated protein kinase C, effected by overnight incubation with 16 μM PMA. Cells were incubated with 10 μM 5-HT for 5 min. Results are from 4–7 paired studies. Control values were 23.8±1.7 for protein kinase C inactivated cells. (**$P < 0.005$ compared with control.)

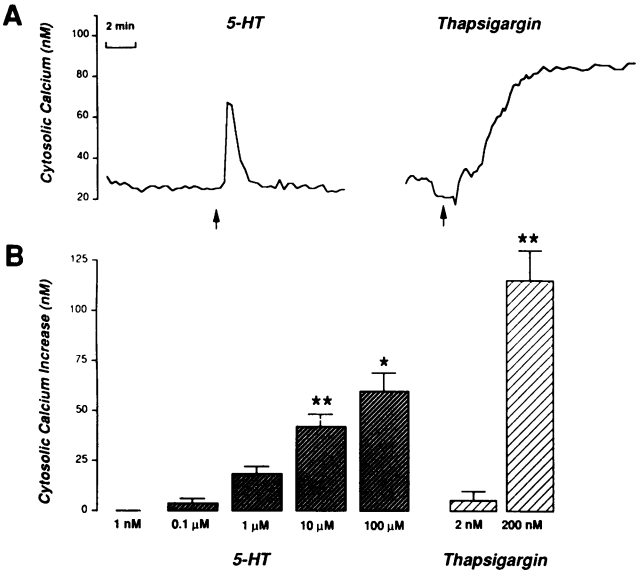

Figure 6. Cytosolic calcium measurement after HA-6 cell stimulation by 5-HT and thapsigargin. Cytosolic calcium was measured in FURA 2-loaded cells using the fluorimetric method described in Methods. (*A*) Typical cytosolic calcium response after exposure to 10 μM 5-HT and 200 nM thapsigargin, using calcium-complete HBSS perfusate buffer. The compounds were injected at the time points indicated by the arrows. (*B*) Dose-response relationships between the same compounds and cytosolic calcium. Results are from 4–8 studies. $^{**}P < 0.005$ compared with basal values.

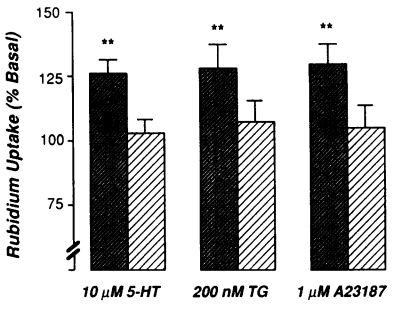

Figure 7. Effects of cytosolic calcium on rubidium uptake in HA-6 cells. The dark bars show the increase in rubidium uptake by 10 μM 5-HT (shown previously in Fig. 1), and by 200 nM thapsigargin (*TG*) and 1 μM A23187 after 10 min incubation in Earle's solution. The light bars represent results after HA-6 cells were incubated for 15 min in calcium-free Earle's solution with 1 mM EGTA, conditions that remove the cytosolic calcium response to 10 μM 5-HT (not shown). Results are from five to eight paired studies. Basal uptake rates were not significantly different after calcium depletion. ▨, Calcium-complete; ▨, calcium-depleted. $^{**}P < 0.01$ compared with unstimulated cells.

quiterpine lactone that increases intracellular calcium without increasing PIP_2 hydrolysis (15–17). The increase in cytosolic calcium following 5-HT was similar to that described for other hormones which increase IP_3 formation (18) and was not removed by the substitution of calcium-free perfusate (not shown). In contrast, thapsigargin caused a less rapid but more sustained increase in cytosolic calcium, as observed in other tissues (15–17). Quantitatively, 10 μM 5-HT increased cytosolic calcium to a peak of 42.2±6.2 nM over baseline ($P < 0.005$; *n*, 8) with an $EC_{50} \sim 3$ μM 5-HT. Thapsigargin increased cytosolic calcium in a dose-responsive manner, with 200 nM leading to a sustained rise of 117.1±15.6 nM calcium ($P < 0.005$; *n*, 6).

Fig. 7 shows the effects of thapsigargin and A23187, a calcium ionophore, on rubidium uptake. Thapsigargin increased rubidium uptake in a dose-responsive fashion (not shown), with 28.2±9.0% stimulation at 200 nM thapsigargin ($P < 0.02$; *n*, 6). A23187 also increased rubidium uptake in a dose-dependent manner, with 1 μM increasing uptake 29.6±7.6% ($P < 0.02$; *n*, 5). In data not shown, 15-min incubation in calcium-free buffer containing 1 mM EGTA removed the cytosolic calcium response to 10 μM 5-HT, presumably due to depletion of cytosolic calcium stores. Fig. 7 shows that these same conditions abolished the effects of 5-HT, thapsigargin, and A23187 on rubidium uptake. These data indicate that the increase in cytosolic calcium following 5-HT_{1A} receptor occupancy appears to be required for the observed stimulation of Na^+/K^+ ATPase.

Fig. 8 shows results from intact cell protein phosphorylation after treatment of HA-6 cells with these same agonists. We showed previously that 5-HT increases the phosphorylation of two cellular proteins: the MARCKS protein, secondary to protein kinase C activation, and another protein with an apparent molecular mass ~ 20 kD (2). Unlike the MARCKS protein, phosphorylation of the smaller protein occurred with 5-HT stimulation of HA-6 cells even after inactivation of protein kinase C, suggesting an alternate signaling pathway (2). Fig. 8 shows that A23187 caused increased phosphorylation of at least two proteins, with apparent mol wt of 22 and 32 kD. The 22-kD phosphoprotein had migration characteristics similar to the smaller protein previously described for 5-HT. The calcium ionophore did not significantly change MARCKS phosphorylation. Thapsigargin also increased phosphorylation of similar smaller protein substrates. In addition, thapsigargin increased MARCKS protein phosphorylation by about 30%, suggesting concomitant protein kinase C activation. Thus, 5-HT, A23187, and thapsigargin increase Na^+/K^+ ATPase activity and increase the phosphorylation of at least two proteins independent of activation of protein kinase C. These proteins and their possible relationship to the changes in Na^+/K^+ ATPase activity need to be clarified, but they suggest the involvement of a calcium-dependent protein kinase.

Discussion

These data demonstrate that agonist occupancy of the 5-HT_{1A} receptor expressed in HeLa cells increases Na^+/K^+ ATPase activity as measured by rubidium uptake, and that cytosolic calcium is required for the response. The transport effect does not require protein kinase C and thus differs from 5-HT stimulation of sodium-dependent phosphate transport, which is mediated by protein kinase C activation (2). Collectively, the data show that a single receptor subtype can independently affect two related transport events via different signaling pathways.

Receptor-mediated breakdown of inositol phospholipids usually leads to simultaneous increases in cytosolic calcium and activation of protein kinase C (19). We have previously shown that the 5-HT_{1A} receptor expressed in HeLa cells is associated with both of these effector systems (2, 4) and causes increased sodium-dependent phosphate uptake via protein kinase C (2). It initially seemed likely that the increase in Na^+/K^+ ATPase activity would have the same mechanism as, and be directly responsible for, the increase in phosphate up-

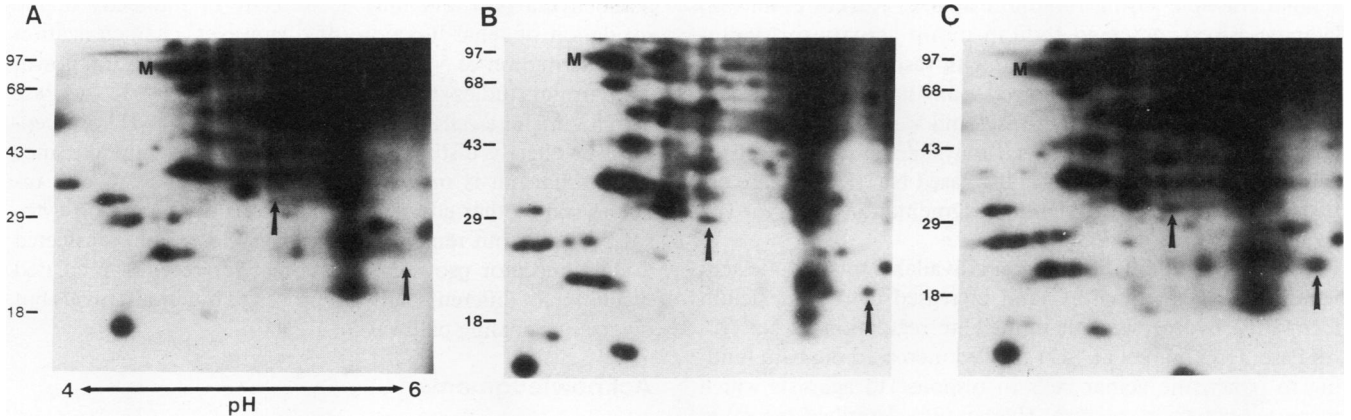

Figure 8. Autoradiograms from intact HA-6 cell protein phosphorylation and two-dimensional gel electrophoresis. Cells were loaded with ^{32}P and treated with agonists for 10 min. The proteins were solubilized and precipitated and samples were prepared for isoelectric focusing as described in the text. The second dimension was performed with 12% SDS-PAGE and the gels were dried and exposed to film. Conditions shown are control (*A*), 1 μM A23187 (*B*), and 200 nM thapsigargin (*C*). A 20-h exposure time was used to improve visualization of the smaller phosphoproteins shown. *M* indicates the MARCKS protein; the *arrows* indicate 32- and 22-kD phosphoproteins. In nonstimulated cells, the MARCKS protein incorporated 710±129, the 32-kD, 68±4, and the 22-kD protein, 65±13 net cpm. Thapsigargin increased MARCKS phosphorylation 250±117 cpm ($P < 0.05$), the 32-kD protein 72±15 cpm ($P < 0.005$), and the 22-kD protein 295±144 cpm ($P = 0.08$). A23187 caused no significant change in MARCKS phosphorylation, but increased phosphorylation of the 32-kD protein 20±14 cpm ($P < 0.05$) and the 22-kD protein 63±26 cpm ($P < 0.05$). Similar results were seen with five experiments.

take. However, the effects of 5-HT on rubidium uptake differ from those on phosphate transport in their lack of protein kinase C involvement. This is supported by two observations: first, various phorbol esters, known short-term protein kinase C activators (19), fail to affect rubidium uptake although they increase phosphate uptake (2); second, downregulation of protein kinase C does not affect the ability of 5-HT to increase Na^+/K^+ ATPase activity although it markedly diminishes 5-HT stimulation of phosphate uptake and eliminates MARCKS protein phosphorylation by both 5-HT and active phorbols (2). These data indicate that the 5-HT_{1A} receptor increases Na^+/K^+ ATPase activity and increases phosphate uptake via different limbs of the phospholipase C pathway.

Inhibition of adenylate cyclase via G_i does not appear to be essential for the increase in Na^+/K^+ ATPase activity since 5-HT increases rubidium uptake even in the presence of dibutyryl cAMP (Fig. 4). These observations for the 5-HT_{1A} receptor differ from those for the 5-HT_{1B} receptor subtype, which causes fibroblast proliferation through adenylate cyclase inhibition (20). However, these findings agree with our previous report for the transfected 5-HT_{1A} receptor and sodium-dependent phosphate transport (2), and they support the physiologic role for additional signaling mechanisms of receptors linked to inhibition of adenylate cyclase (21, 22).

5-HT_{1A} receptor activation causes unique changes in the kinetic properties of Na^+/K^+ ATPase. Data transformation using the Hill equation reinforces two primary observations. First, the changes in kinetic parameters of rubidium uptake are qualitatively different for 5-HT treatment and protein kinase A activation. Protein kinase A increases the V_{max} for rubidium uptake presumably through effects on sodium channels (23) or sodium-proton exchange (24). Second, the kinetic characteristics of stimulation of Na^+/K^+ ATPase are fundamentally different for 5-HT and nystatin, suggesting a lack of involvement of intracellular sodium concentration in receptor-mediated stimulation of Na^+/K^+ ATPase. An alternate explanation for 5-HT-mediated stimulation of Na^+/K^+ ATPase could be increased sodium–calcium exchange. This seems unlikely because the time courses for the changes in cytosolic calcium and rubidium uptake are different, and because increased sodium–calcium exchange would likely affect the V_{max} for rubidium uptake (25). In addition, the relationship between cytosolic calcium concentration and stimulation of Na^+/K^+ ATPase is not simple and direct. For example, thapsigargin causes a large increase in cytosolic calcium but a similar level of Na^+/K^+ ATPase stimulation. The kinetic characteristics of 5-HT_{1A} receptor stimulation of Na^+/K^+ ATPase suggest that there is direct modification of the enzyme complex.

Recent work proposes a biochemical framework for alteration of Na^+/K^+ ATPase by classic cell messenger systems. Na^+/K^+ ATPase exists as a complex of large molecular weight α-subunits and smaller glycosylated β-subunits (26, 27). At least three distinct forms of the α-subunit have been cloned and sequenced (26, 27), and a potential cAMP-dependent protein kinase phosphorylation site is conserved in the primary structure of all three isoforms. Although agonist-induced changes which occur over the long term have been studied extensively (28–30), remarkably little information is available regarding short-term regulation.

Receptor-mediated rapid regulation of Na^+/K^+ ATPase has been proposed for arginine vasopressin (31, 32), dopamine (33–35), and catecholamines (31, 36, 37), presumably through activation of intracellular signals. Studies in intact cells invoke protein kinase A (38–40), protein kinase C (33–35, 41), and calcium/calmodulin-dependent protein kinases (37), but frequently these studies fail to account for concurrent changes in cytosolic concentrations of either sodium or ATP. Most importantly, direct biochemical evidence for α-subunit phosphorylation by a protein kinase is still lacking (26). Giesen et al. (38) described decreased Na^+/K^+ ATPase enzyme activity with cAMP and cAMP analogues in a crude preparation of rat kidney. Lingham and Sen (40) correlated inhibition of rat brain sodium pump activity with protein kinase A activity and identified a possible intermediate phosphoprotein from a sub-

cellular fraction. Using rubidium uptake, Reznik et al. (32) later described increased sodium pump activity in Madin-Darby canine kidney cells treated with vasopressin, an agonist shown to activate adenylate cyclase in those cells. However, the effect required 4–6 h for onset and was not clearly mimicked by cAMP analogues. Electrophysiologic study of retinal epithelium showed that cAMP increased Na^+/K^+ ATPase activity within minutes and that the augmentation lasted for 1–2 h (39).

More compelling information is available to associate activation of protein kinase C and increased cytosolic calcium from PIP_2 hydrolysis with immediate regulation of Na^+/K^+ ATPase. Hootman et al. (41) showed increased ouabain binding to pancreatic acinar cells in response to agonists which increased cytosolic calcium. Henley (36) described increased hepatocyte pump activity in response to epinephrine, apparently requiring the presence of extracellular calcium. Other studies have supported the role of the calcium signal, though they differ with regard to the origin of the calcium (31, 37). Lynch et al. (37) dissociated the effect of agonists on intracellular calcium from that on pump activity in hepatocytes, but showed a close dose-response association between agonist, IP_3 generation, and stimulation of pump activity. They also showed activation of Na^+/K^+ ATPase by phorbol esters, implicating protein kinase C involvement. Recent studies on proximal renal tubules demonstrated inhibition of pump activity by dopamine, apparently through a PTx-sensitive G protein, and reproduction of this effect by activation of protein kinase C (33–35). These observations provide ample precedent for the short-term regulation of Na^+/K^+ ATPase by receptor-associated events but in general they lack systematic investigation of the full sequence from occupancy of a defined receptor to biologic effect.

By characterizing the regulation of Na^+/K^+ ATPase and of sodium-dependent phosphate transport by the transfected 5-HT_{1A} receptor, our recent studies demonstrate both a functional and a biochemical distinction between activation of protein kinase C and increase in cytosolic calcium. Our previous studies demonstrate that 5-HT induces phosphorylation of proteins via mechanisms that are both dependent (MARCKS protein) and independent (approximately 20 kD protein) of protein kinase C activation (2). The present studies confirm and extend this information by showing that A23187 and thapsigargin also increase the phosphorylation of at least two other proteins, presumably via a currently uncharacterized calcium-dependent protein kinase. Pollack has described sodium and ATP-dependent phosphorylation of a 93-kD catalytic subunit in HeLa cells (42), but a similar presumed protein kinase substrate was not observed in our phosphorylation experiments. Our results are comparable to those from hepatocytes where the phosphorylation of several distinct proteins has been linked to increases in cytosolic calcium (43) and to the findings of Thastrup et al. (17) who suggested a specific phosphoprotein pattern following thapsigargin treatment of platelets. Incidentally, thapsigargin also increases MARCKS protein phosphorylation thereby indicating increased protein kinase C activity. Thus our data indicate that the action of 5-HT on Na^+/K^+ ATPase involves a calcium-dependent signaling process different from protein kinase C although they do not identify the specific protein kinase or substrate.

The physiologic implications of these observations are uncertain. Serotonin is present in high concentrations in renal parenchyma (44) and may act directly or indirectly in the regulation of renal function (45). However, characterization and distribution of 5-HT receptors in the kidney is unknown. The current studies show that 5-HT increases Na^+/K^+ ATPase activity in HeLa cells transfected to express the 5-HT_{1A} receptor. The effect is distinct from that on phosphate uptake since the mechanism is independent of protein kinase C and involves some other effector system that is dependent on cytosolic calcium but remains otherwise unclear. The transfected 5-HT_{1A} receptor provides a paradigm for receptor-mediated regulation of different transport processes by simultaneous but divergent signaling pathways.

Acknowledgments

We are grateful for the editorial assistance and the kind donation of HA-6 cells by Dr. Annick Fargin, and the laboratory support of Dr. Robert J. Lefkowitz and Dr. Perry J. Blackshear. Thapsigargin was supplied by Dr. Peter Anderson as a gift from Dr. Ole Thastrup. We appreciate the technical support of LaRue D. Poe, G. G. Collinsworth, and D. M. Haupt.

Supported in part by National Institutes of Health grant DK-7308 Haskel Schiff Research Fund, and a Department of Veterans Affairs Merit Review Award.

References

1. Schmidt, W., and S. J. Peroutka. 1989. 5-Hydroxytryptamine receptor "families." *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 3:2242–2249.

2. Raymond, J. R., A. Fargin, J. P. Middleton, J. M. Graff, D. M. Haupt, M. G. Caron, R. J. Lefkowitz, and V. W. Dennis. 1989. The human 5-HT_{1A} receptor expressed in HeLa cells stimulates sodium-dependent phosphate uptake via protein kinase C. *J. Biol. Chem.* 264:21943–21950.

3. Fargin, A., J. R. Raymond, M. J. Lohse, B. K. Kobilka, M. G. Caron, and R. J. Lefkowitz. 1988. The genomic clone G-21, which resembles a β-adrenergic receptor sequence encodes the 5-HT_{1A} receptor. *Nature (Lond.).* 335:358–360.

4. Fargin, A., J. R. Raymond, J. W. Regan, S. Cotecchia, R. J. Lefkowitz, and M. G. Caron. 1989. Effector coupling of the cloned 5-HT1A receptor. *J. Biol. Chem.* 264:14848–14852.

5. Rosenthal, N. 1987. Identification of regulatory elements of cloned genes with functional assays. *Methods Enzymol.* 152:704–720.

6. Miyamoto, H., T. Ikehama, H. Yamaguchi, K. Hosokawa, and T. Yonezu. 1986. Kinetic mechanism of Na^+ K^+ Cl^- cotransport as studied by Rb^+ influx into HeLa cells. Effects of extracellular monovalent ions. *J. Membr. Biol.* 92:135–50.

7. Ikehara, T., H. Yamaguchi, T. Sakai, and H. Miyamoto. 1984. Kinetic parameters and mechanism of active cation transport in HeLa cells as studied by Rb^+ influx. *Biochim. Biophys. Acta.* 775:297–307.

8. Blackshear, P. J., R. A. Nemenoff, and J. Avruch. 1983. Preliminary characterization of a heat-stable protein from rat adipose tissue whose phosphorylation is stimulated by insulin. *Biochem. J.* 204:817–824.

9. Stumpo, D. J., J. M. Graff, K. A. Albert, P. Greengard, and P. J. Blackshear. 1989. Molecular cloning, characterization, and expression of a cDNA encoding the "80- to 87-kDa" myristoylated alanine-rich C kinase substrate: a major cellular substrate for protein kinase C. *Proc. Natl. Acad. Sci. USA.* 86:4012–4016.

10. Grynkiewicz, G., M. Poenie, and R. Y. Tsien. 1985. A new generation of Ca^{2+} indicators with improved fluorimetric properties. *J. Biol. Chem.* 260:3440–3450.

11. Schoeffter, P., and D. Hoyer. 1988. Centrally acting hypotensive agents with affinity for 5-HT_{1A} binding sites inhibit forskolin-stimulated adenylate cyclase activity in calf hippocampus. *Br. J. Pharmacol.* 95:975–985.

12. Doods, H. N., H. W. Boddeke, H. O. Kalkman, D. Hoyer, M. Mathy, and P. A. van Zwieten. 1988. Central 5-HT_1A receptors and the mechanism of the central hypotensive effect of 8-(OH)-DPAT, DP-5-CT, R28935, and uradipil. *J. Cardiovasc. Pharmacol.* 11:432–437.

13. Segel, I. H. 1987. Enzyme kinetics: behavior and analysis of rapid equilibrium and steady-state enzyme systems. John Wiley & Sons, Inc., New York. 353 pp.

14. Soltoff, S. P., and L. J. Mandel. 1984. Active ion transport in the renal proximal tubule. I: Transport and metabolic studies. *J. Gen. Physiol.* 81:601–622.

15. Takemura, H., A. R. Hughes, O. Thastrup, and J. W. Putney. 1989. Activation of calcium entry by the tumor promoter thapsigargin in parotid acinar cells. *J. Biol. Chem.* 264:12266–12271.

16. Jackson, T. R., S. I. Patterson, O. Thastrup, and M. R. Hanley. 1988. A novel tumor promoter, thapsigargin, transiently increases cytoplasmic free Ca^{2+} without generation of inositol phosphates in NG115-401L neuronal cells. *Biochem. J.* 253:81–86.

17. Thastrup, O., H. Linnebjurg, P. J. Bjerrum, J. B. Knudsen, and S. B. Christensen. 1987. The inflammatory and tumor-promoting sesquiterpine lactone, thapsigargin, activates platelets by selective mobilization of calcium as shown by protein phosphorylations. *Biochim. Biophys. Acta.* 927:65–73.

18. Berridge, M. J., and R. F. Irvine. 1989. Inositol phosphates and cell signaling. *Nature (Lond.).* 341:197–205.

19. Nishizuka, Y. 1984. The role of protein kinase C in cell surface signal transduction and tumor promotion. *Nature (Lond.).* 308:693–698.

20. Seuwen, K., I. Magnaldo, and J. Pouyssegur. 1988. Serotonin stimulates DNA synthesis in fibroblasts acting through 5-HT_{1B} receptors coupled to a G_i-protein. *Nature (Lond.).* 335:254–256.

21. Limbird, L. E. 1988. Receptors linked to inhibition of adenylate cyclase: additional signaling mechanisms. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 2:2686–2695.

22. Ashkenazi, A., J. W. Winslow, E. G. Peralta, G. L. Peterson, M. I. Schimerlik, D. L. Capon, and J. Ramachandran. 1987. An M2 muscarinic receptor receptor subtype coupled to both adenylyl cyclase and phosphoinositide turnover. *Science (Wash. DC).* 238:672–675.

23. Costa, M. R. C., and W. A. Catteral. 1984. Cyclic AMP-dependent phosphorylation of the α subunit of the sodium channel in synaptic nerve ending particles. *J. Biol. Chem.* 259:8210–8218.

24. Pollock, A. S., D. G. Warnock, and G. J. Strewler. 1986. Parathyroid hormone inhibition of Na^+-H^+ antiporter activity in a cultured renal cell line. *Am. J. Physiol.* 250:F217–F225.

25. Stein, W. D. 1967. The Movement of Molecules across Cell Membranes. Academic Press, Inc., New York. 177–206.

26. Shull, G. E., J. Greeb, and J. B. Lingrel. 1986. Molecular cloning of three distinct forms of the Na^+, K^+-ATPase α-subunit from rat brain. *Biochemistry.* 25:8125–8132.

27. Shull, G. E., L. K. Lane, and J. B. Lingrel. 1986. Amino-acid sequence of the beta-subunit of the (Na^+ + K^+) ATPase deduced from a cDNA. *Nature (Lond.).* 321:429–431.

28. Edelman, I. S., T. A. Pressley, and A. Hiatt. 1985. Regulation of mammalian Na, K-ATPase. *In* The Sodium Pump. The Company of Biologists, Ltd., Cambridge, UK. 153–159.

29. Gick, G. G., F. Ismail-Beigi, and I. S. Edelman. 1988. Overview: Hormonal regulation of Na, K-ATPase. *In* The Na^+-K^+-Pump. Part B: Cellular Aspects. J. Skou, J. Norby, A. Mansbach, and M. Esman, editors. Alan R. Liss, Inc., New York. 353–356.

30. Katz, A. 1988. Overview: Role of Na-K-ATPase in kidney function. *In* The Na^+-K^+-Pump. Part B: Cellular Aspects. J. Skou, J. Norby, A. Mansbach, and M. Esman, editors. Alan R. Liss, Inc., New York. 207–232.

31. Berthon, B., T. Capiod, and M. Claret. 1985. Effects of noradrenaline, vasopressin and angiotensin on the Na-K pump in rat isolated liver cells. *Br. J. Pharmacol.* 86:151–161.

32. Reznik, V. M., R. J. Shapiro, and S. A. Mendoza. 1985. Vasopressin stimulates DNA synthesis and ion transport in quiescent epithelial cells. *Am. J. Physiol.* 249:C267–C270.

33. Aperia, A., A. Bertorello, and I. Seri. 1987. Dopamine causes inhibition of Na^+-K^+-ATPase activity in rat proximal convoluted tubule segments. *Am. J. Physiol.* 252:F39–F45.

34. Bertorello, A., and A. Aperia. 1989. Regulation of Na^+-K^+-ATPase activity in kidney proximal tubules. Involvement of GTP binding proteins. *Am. J. Physiol.* 256:F57–62.

35. Bertorello, A., and A. Aperia. 1989. Na^+-K^+-ATPase is an effector protein for protein kinase C in renal proximal tubule cells. *Am. J. Physiol.* 256:F370–373.

36. Henley, J. M. 1985. Epinephrine-stimulated maintained rubidium efflux from guinea pig hepatocytes may involve alpha1- and alpha2-adrenoreceptors. *Mol. Pharmacol.* 28:431–35.

37. Lynch, C. J., P. B. Wilson, P. F. Blackmore, and J. H. Exton. 1986. The hormone-sensitive hepatic Na^+-pump. *J. Biol. Chem.* 261:14551–14556.

38. Giesen, E. M., J. L. Imbs, M. Grima, M. Schmidt, and J. Schwartz. 1984. Modulation of renal ATPase activities by cyclic AMP. *Biochem. Biophys. Res. Commun.* 120:619–624.

39. Hughes, B. A., S. S. Miller, D. P. Joseph, and J. L. Edelman. 1988. cAMP stimulates the Na^+-K^+ pump in frog retinal pigment epithelium. *Am. J. Physiol.* 254:C84–C98.

40. Lingham, R. B., and A. K. Sen. 1982. Regulation of rat brain (Na^+ + K^+)-ATPase activity by cyclic AMP. *Biochim. Biophys. Acta.* 688:475–485.

41. Hootman, S. R., S. R. Ernst, and J. A. Williams. 1983. Secretagogue regulation of Na^+-K^+ pump activity in pancreatic acinar cells. *Am. J. Physiol.* 245:6339–346.

42. Pollack, L. R., E. H. Tate, and J. S. Cook. 1981. Turnover and regulation of Na-K-ATPase in HeLa cells. *Am. J. Physiol.* 241:C173–183.

43. Garrison, J. C., D. E. Johnsen, and C. P. Campanile. 1984. Evidence for the role of phosphorylase kinase, protein kinase C and other Ca^{2+}-sensitive protein kinases in the response of hepatocytes to angiotensin II and vasopressin. *J. Biol. Chem.* 259:3283–3292.

44. Fischer, C. A., and M. H. Aprison. 1972. Determination of nanomole levels of 5-hydroxytryptophan, 5-hydroxytryptamine, and 5-hydroxyindoleacetic acid in the same sample. *Anal. Biochem.* 46:67–84.

45. Morgan, D. A., P. Thoren, E. A. Wilczynski, R. G. Victor, and A. L. Mark. 1988. Serotonergic mechanisms mediate renal sympathoinhibition during severe hemorrhage. *Am. J. Physiol.* 255:H496–H502.