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Research Article

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Regulation of Hormone-sensitive Calcium Influx by the Adenylyl Cyclase System in Renal Epithelial Cells

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Abstract

To study signaling pathways regulated by α_s and α_{11} in renal epithelial cells, we expressed mutant, activated forms of α_s and α_{11} in a continuous proximal tubule cell line (MCT cells). α_{sQ227L} increased cAMP production, and $\alpha_{11Q204L}$ reduced forskolin-sensitive cAMP production. $\alpha_{11Q204L}$ increased and α_{sQ227L} decreased bradykinin-induced Ca influx across the cell membrane, but neither mutant affected bradykinin-stimulated intracellular Ca release or basal Ca influx. Bradykinin-stimulated Ca influx was reduced by dibutyryl cAMP, isoproterenol, and forskolin. Expression of a mutant regulatory type I subunit for cAMP-dependent protein kinase with reduced affinity for cAMP and treatment with KT-5720, a specific cAMP-dependent protein kinase inhibitor, enhanced Ca influx to a degree similar to that in cells expressing $\alpha_{11Q204L}$. Bradykinin-stimulated *c-fos* mRNA expression is partially dependent on extracellular Ca. α_{sQ227L} reduced and $\alpha_{11Q204L}$ enhanced bradykinin-stimulated *c-fos* expression. Consequently, in bradykinin-stimulated cells, the adenylyl cyclase system regulates Ca influx through cAMP-dependent protein kinase, but not intracellular Ca release. Furthermore, the Ca influx mechanism acts as an integrator of two signaling pathways such that Ca-dependent signals are damped by activators of adenylyl cyclase and enhanced by inhibitors of adenylyl cyclase. (*J. Clin. Invest.* 1994. 94:328–336.) Key words: G protein • adenylyl cyclase • intracellular calcium • membrane signal transduction

Introduction

The heterotrimeric G proteins are a family of proteins that couple receptors on the outside of cells to effector systems such as enzymes or ion channels that alter the intracellular environment (1–4). Effectors can be regulated by G proteins at multiple levels including direct interaction and by second messenger-dependent protein kinases. Despite considerable effort, the effector systems regulated by specific G proteins have not been defined fully in most cases for a number of reasons. Many ligands for G protein-coupled receptors such as catecholamines, acetylcholine, parathyroid hormone, and angiotensin II

appear to activate multiple G proteins, so that the contribution of individual members of the G protein family to signaling pathways is difficult to determine. The individual components are difficult to study because they are expressed in a tissue-specific manner at low levels. Reconstitution studies demonstrate that an individual G protein may be capable of participating in interactions with a variety of receptors and effectors, but the specific makeup of a signaling pathway depends on its cellular context. Consequently, signaling pathways in cells such as renal epithelial cells, fibroblasts, and excitable cells are likely to be different.

Most evidence indicates that α subunits carry the specificity for receptor and effector interactions, although roles for $\beta\gamma$ subunits in effector regulation are being defined (5–8). G proteins are classified according to their α chains and can be grouped into families according to their structures (9). α_s regulates adenylyl cyclase in all tissues studied, and in cardiac myocytes appears to regulate Ca channels through second messenger-dependent protein kinases and possibly a direct membrane-delimited system (10, 11). The mammalian G protein α_i family includes α_{11} – α_{13} , α_o , α_2 , and the transducins (9). These proteins are all products of distinct genes, yet are highly homologous. With the exception of the transducins that are expressed exclusively in the retina, knowledge of the function of individual members of this family is incomplete (1–4, 12). α_{11} – α_{13} and α_o are pertussis toxin substrates and consequently are candidates for mediating processes that are guanine nucleotide-dependent and sensitive to inhibition by pertussis toxin. The processes potentially regulated by the pertussis toxin substrates include inhibition of hormone-stimulated adenylyl cyclases, stimulation of phospholipase C, regulation of phospholipase A₂, and K, Ca, and Na channels (1–4, 10, 11).

Intracellular Ca can be regulated by a variety of G protein-dependent and -independent mechanisms including release from intracellular stores [usually in response to phospholipase C activation and inositol triphosphate (IP₃)¹ production], Ca influx across the plasma membrane through a variety of Ca channels, and active transport out of the cytosol by Ca-ATPases (13–15). The Ca influx pathways in excitable tissues, neurons, and muscle cells are better defined structurally and pharmacologically than their counterparts in other tissues such as epithelial cells.

To study the effects of α_{11} and α_s on regulation of the adenylyl cyclase(s) expressed in renal epithelial cells and intracellular Ca, along with possible interactions of these systems, we expressed mutant, GTPase-deficient forms of α_{11} and α_s in a renal epithelial cell line using a regulatable metallothionein promoter (16, 17). This system allowed us to study the effects

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1. Abbreviations used in this paper: db-cAMP, dibutyryl cAMP; IBMX, 3-isobutyl-1-methyl xanthine; IP₃, inositol triphosphate; Neo, neomycin-resistant; PK, protein kinase.

of each α chain individually and the effects of increased and decreased adenylyl cyclase activity. We chose this expression system in order to avoid high level expression of activated proteins. In these studies, we demonstrate that the mutant α_s stimulates and the mutant α_{i1} inhibits adenylyl cyclase. We identify a new bradykinin-sensitive Ca influx pathway in which activity is modulated by the adenylyl cyclase system through cAMP-dependent protein kinase (PK A). Ca influx is inversely related to adenylyl cyclase activity regulated by the expressed α chains such that Ca-dependent signals are modulated by the adenylyl cyclase system in MCT cells. A Ca influx pathway that is inhibited by the adenylyl cyclase system has not been described previously.

Methods

Materials. Restriction and DNA-modifying enzymes were obtained from Promega Biotech (Madison, WI) and radionucleotides from DuPont-New England Nuclear (Boston, MA). Fura-2 was purchased from Molecular Probes (Eugene, OR) and KT-5720 from LC Laboratories (Woburn, MA). Tissue culture medium, serum, and synthetic peptides (Kemptide and PK A inhibitor) were purchased from Gibco Laboratories (Grand Island, NY). Tissue culture plasticware was from Falcon. Other chemicals were from Sigma Chemical Co. (St. Louis, MO) or molecular biology grade from Fisher Scientific Co. (Fair Lawn, NJ).

Cell culture. MCT cells (an SV40-transformed mouse proximal tubule cell line) were obtained from Eric Neilson, University of Pennsylvania Medical School (18). The cells were grown in a variety of plastic tissue culture plates and were maintained in RPMI 1640 with 5% fetal calf serum (Gibco Laboratories). Medium for cell lines expressing mutant α chains or the vector alone also contained G-418 (Gibco Laboratories), 250 μ g/ml. 24-h before experiments, cells were serum deprived, and G-418 was removed. Where indicated, CdCl₂ (1 μ M) was present in the medium for 8 h. This protocol resulted in maximal induction of mRNA for the mutant α chains. All cells were grown in 5% CO₂, 95% air incubators at 37°C.

Mutagenesis. Rat cDNAs coding for α_s (the 52-kD form) and α_{i1} were obtained from Randall Reed, Johns Hopkins University Medical School (19). Point mutations changing Q227 to L in α_s and Q204 to L in α_{i1} were made using the Bio-Rad Laboratories (Richmond, CA) Phagemid Mutagen kit (20). The cDNAs coding for the α chains were subcloned into PTz19u (Bio-Rad Laboratories) using HindIII sites for α_s and EcoRI sites for α_{i1} . Plasmids containing the α chains were transformed into *Escherichia coli* CJ236, and single-stranded DNA containing uracil in place of thymidine was produced by infection of the bacteria with helper phage. The mutagenic oligonucleotides, bp 852–871 for α_s (5'-GTGGGCGCCTTCGCGATGAA-3') and bp 818–839 for α_{i1} (5'-CGTGGGAGGCCTTAGATCAGAGCG-3'), were annealed to their respective single-stranded templates and were incubated with dNTPs (0.4 mM), DNA polymerase, and T4 DNA ligase in buffer containing (mM) 23 Tris (pH 7.9), 5.0 MgCl₂, 35 NaCl, 1.5 DTT, and 0.75 ATP for 5 min on ice, at 25°C for 5 min, and at 37°C for 90 min. The reaction mix was then transformed into *E. coli* MV-1190, and colonies were selected on ampicillin plates. Double-stranded sequencing was performed on DNA purified from individual colonies, to document the presence of mutations (20).

DNA constructions. The expression vector Zem-228, which contains a mouse metallothionein (MT-1) promoter, an SV40 poly A sequence, and the gene for G-418 resistance, was obtained from Stan McKnight, University of Washington Medical School (The construction or use of Zem-228 has not been published, but the MT-1 promoter used in it is the same as that described in reference 21). The mutagenized α chains, α_{sQ227L} and $\alpha_{i1Q204L}$, were subcloned into Zem-228 at the unique BamHI cloning site using blunt end ligation. Rev B (the mutant type I regulatory subunit for cAMP-dependent protein kinase) was obtained from Stan McKnight and used in the vector described in reference 21.

Mammalian cell expression. 15 μ g of plasmid DNA [Zem-228 without insert, α_{sQ227L} , $\alpha_{i1Q204L}$, and Rev B (the mutant protein kinase A type I regulatory subunit in a similar vector)] was transfected into MCT cells using lipofectin reagent (Bethesda Research Laboratories, Bethesda, MD). The Rev B plasmid, which does not contain a neomycin-resistance gene, was co-transfected with 1.5 μ g of pSV2-Neo. The cells were grown for 48 h, and neomycin-resistant clones were selected in 500 μ g/ml G-418 (Gibco Laboratories) by limiting dilution. Expression of cDNAs was documented by RNA blots, immunoblots, and demonstration of the appropriate biochemical phenotype.

Immunoblots. Cell extracts were prepared by lysis of whole cell monolayers in 2% SDS. Protein content was normalized by Lowry protein assay (with SDS in the standards) (22), and 50 μ g of cell extract was size fractionated on 9% SDS-polyacrylamide gels and electrotransferred to nitrocellulose. The blots were blocked in blotto (powdered nonfat milk, 5%; 50 mM Tris pH 7.4; 150 mM NaCl; 5.0 mM EDTA; 0.01% Na Azide; and 0.05% Tween 20) and incubated in blotto with the primary antibodies. The antibody to α_s that recognizes the unique region of α_s (amino acids 323–339) and was produced in the Bourne laboratory, was obtained from Henry Bourne (17). The α_i antibody P960 was obtained from Suzanne Mumby (23). The blots were washed five times in blotto and incubated with the secondary antibody, ¹²⁵I-labeled goat anti-rabbit IgG Fab (New England Nuclear, Boston, MA), washed five times in blotto, air dried, and exposed to film.

RNA blots. Total RNA was prepared from cells grown in 100-mm dishes by the guanidinium thiocyanate method followed by CsCl centrifugation. 10 μ g of total RNA was size fractionated on 0.8% formaldehyde-agarose gels, transferred to a nylon membrane (Gene Screen; New England Nuclear), and probed (20). Probes for the RNA blots were as follows: rat α_s , the 1,389-bp HindIII fragment that contains the entire coding region; for rat α_{i1} , the XbaI-EcoRI fragment that contains 600 bp of the 3' UTR; for rat glyceraldehyde-3-phosphate dehydrogenase, 1,380-bp PstI-PstI fragment that contains the entire coding region (18); and for *c-fos*, the full length rat cDNA (a gift of Dr. T. Curran) (19, 24, 25). The probes were labeled with [³²P]dATP by the random primer method (Primagene; Promega).

cAMP production measurements. cAMP production was measured as [³H]cAMP formation after incubation of cells overnight in serum-free medium with 2 μ Ci/ml [³H]adenine (26, 27). For measurement of intracellular cAMP, the cells were washed twice with PBS and incubated in 1.0 ml of serum-free medium containing 2.0 mM 3-isobutyl-1-methyl xanthine (IBMX) and hormones or activators as indicated. After 30 min in the incubator, the reaction was terminated by aspiration of the medium and addition of 1.0 ml of 10% TCA. cAMP production was linear for 45 min. The cells were held at 4°C for 30 min, the medium was removed, and [³H]cAMP was isolated by column chromatography by the method of Salomon et al. (28). The stop solution (10% TCA) contained ~ 800 cpm/ml of [³²P]cAMP to correct for variable recovery by individual columns. The precipitated protein was dissolved in 1.0 ml of 1.0 M NaOH and assayed for protein by the method of Lowry et al. (23). Where indicated, pertussis toxin was added to cell culture medium at a concentration of 200 ng/ml for 8 h before the production assay. All points are the mean of triplicate samples and are expressed as cpm [³H]cAMP/min per mg protein \pm SEM.

Intracellular Ca measurements. Intracellular Ca was measured fluorometrically in Fura-2-loaded cells in suspension. Approximately 5 \times 10⁷ cells were trypsinized with EDTA, washed twice with uptake medium [(mM) 140 NaCl; 5.0 KCl; 1.0 MgCl₂; 10 HEPES, pH 7.4; 1.0 mg/ml BSA and 2.0 mg/ml glucose] and resuspended and incubated in 2.0 ml of uptake medium containing 2.5 μ M Fura-2-AM for 30 min at 35°C. The cells were washed again and resuspended in 2.0 ml of uptake medium. 50 μ l, or ~ 10⁶ cells, was diluted into 2.0 ml of uptake medium at 37°C with constant stirring for studies (29).

Ca measurements were made in a LS-5B spectrometer or an SLM 8000C fluorimeter (Perkin-Elmer Cetus Instruments, Norwalk, CT) (29). Excitation was at 340 nm and emission at 500 nm. At the end of studies, Ca measurements were calibrated by lysing the cells with digitonin (50 μ g/ml) and 2.0 mM Ca to obtain maximum fluorescence (F_{max})

and then adding 10 mM EDTA (above pH 8.15) to obtain minimum fluorescence (F_{\min}). Intracellular Ca was calculated according to the method of Tsien (30).

PKA activity assay. PKA activity was measured as described by Clegg et al. (21) using Kempptide (LRRASLG) as a synthetic substrate (21). Cells were washed in PBS, scraped in isotonic buffer [(mM) 30 Hepes, pH 7.4; 250 sucrose; 0.1 PMSF; 1 pepstatin A]. The cells were centrifuged, resuspended in homogenization buffer [(mM) 10 Na PO₄, pH 7.0; 1 EDTA; 1 DTT; 250 sucrose; 0.1 PMSF; 1 pepstatin A; and 0.5 IBMX], and disrupted by sonication. The particulate fraction was removed by centrifugation and the supernatant saved and assayed for protein content. Approximately 10 μ g of protein was used for each assay. To measure specific kinase activity, the activity of samples incubated with PKI (TYADFIASGRTGRRN, a specific peptide inhibitor of PKA) in addition to Kempptide was subtracted from the activity of samples incubated with Kempptide alone. Cell extracts were assayed under basal conditions and in the presence of cAMP (10^{-5} M) in duplicate for 5 min during which time the assay was linear. The assay buffer contained 200 μ M ATP, 10 mM Mg acetate, 30 μ M Kempptide, 20 mM Tris, 0.5 mM IBMX, 10 mM DTT, and 0.00225 mCi [γ -³²P]ATP per tube in a final volume of 50 μ l. Reactions were stopped by spotting 25 μ l from each tube on phosphocellulose and rapid washing of them in 75 mM phosphoric acid. Samples were assayed in duplicate and repeated three times with similar results.

Statistical analysis. Experimental groups were compared by ANOVA using the InStat biostatistics program from GraphPad Software (San Diego, CA). Multiple comparisons among groups were made using the Student-Newman-Keuls test. *P* values < 0.05 were considered significant.

Results

Selection of clones expressing mutant α chains. Clonal G-418-resistant cell lines transfected with α_{sQ227L} , $\alpha_{i1Q204L}$, and the vector without insert (Zem-228) were obtained by limiting dilution. Cell lines were screened for expression of α_{sQ227L} by RIA for cAMP and chosen on the basis of increased basal and a Cd-dependent increase in cAMP production. Eight potential clones expressing α_{sQ227L} were screened, seven of which were positive. Clones transfected with $\alpha_{i1Q204L}$ were screened for expression of the cDNA by RNA blot (see below). Six potential clones expressing $\alpha_{i1Q204L}$ were screened. Five of these clones were positive for $\alpha_{i1Q204L}$ mRNA and inhibition of forskolin-stimulated cAMP production. The cell line with the best regulation of adenylyl cyclase activity by Cd in each group was selected for further study.

Expression of mutant α chain cDNAs and protein. Successful expression of the cDNAs in MCT cells was shown by RNA blot in which unique mRNAs of 1.55 kb (corresponding to α_{sQ227L}) and 2.15 kb (corresponding to $\alpha_{i1Q204L}$) were present and regulated by Cd (data not shown). Immunoblots of extracts from cells expressing the mutant α chains using antisera for α_s or the α_s demonstrate increased antigen in the respective cell lines compared to wild-type or neomycin-resistant (neo) control cells (Fig. 1 A and B). The first lane of both immunoblots contains cholate extracts of rat brain (60 μ g of protein) as G protein α chain standards. Proteins in these lanes migrate slightly faster than the lanes containing MCT cell extracts because they contain cholate rather than SDS. Fig. 1 A shows an immunoblot of extracts of wild-type, neomycin-resistant control cells and cells expressing α_{sQ227L} with an antibody to α_s (17). In all cell lines without and with Cd, bands at 45 and 52 kD are seen that correspond to the splice variants of α_s . In the cells expressing α_{sQ227L} , in the presence of Cd a large increase in

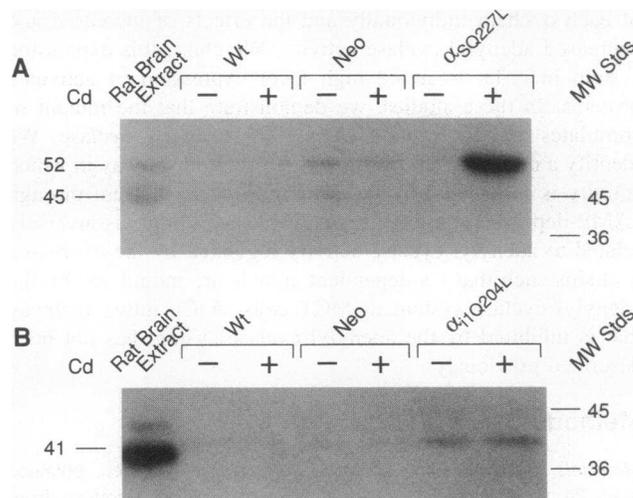


Figure 1. Immunoblots of MCT cell extracts using antibodies to α_s and α_i1 . The cells were treated without and with CdCl₂ as described in Methods. 50 μ g of extract protein were loaded in each lane except for lane 1 labeled Rat brain extract, which contains 60 μ g of cholate extract. (A) Wild-type (Wt), neomycin-resistant control cells (Neo) and cells expressing α_{sQ227L} blotted with an antibody to α_s . (B) A similar immunoblot but with cells expressing $\alpha_{i1Q204L}$ blotted with antibody P960 that recognizes α_i1 .

antigen at 52 kD is seen that corresponds to the mutant protein and correlates with mRNA levels. Fig. 1 B shows an immunoblot of wild-type and neomycin-resistant cells and cells expressing $\alpha_{i1Q204L}$ without and with Cd pretreatment. In this case, the antibody used was p960 which recognizes the α_i1 , α_i2 , α_i3 , and other α chains (23). In the cells expressing $\alpha_{i1Q204L}$, an increase in antigen at 41 kD is seen that corresponds to the expressed protein. As is the case for the RNA blot, the basal level of expression of the mutant α_i1 is higher and increases less with Cd pretreatment than the mutant α_s .

Adenylyl cyclase phenotypes. The mutations in both α chains reduce their GTPase activity and cause persistent activation by maintaining them in the GTP-bound conformation (16, 17, 27, 31, 32). Under normal conditions in the cell, both α_s and α_i1 have significant rates of spontaneous guanine nucleotide exchange, but remain inactive in the presence of GTP because the rate of GTP hydrolysis is more rapid than their nucleotide exchange rates (2, 33, 34). However, the two mutant α chains (α_{sQ227L} and $\alpha_{i1Q204L}$) are in their active forms in the presence of normal intracellular GTP concentrations because of their very slow rate of GTP hydrolysis. The ability of hormones to regulate cAMP production is altered by expression of $\alpha_{i1Q204L}$ (Fig. 2). Cd pretreatment of cells was not used in these studies because even in the absence of Cd, the cAMP production is maximally inhibited by $\alpha_{i1Q204L}$ and is measurably increased by α_{sQ227L} (Fig. 2).

In control (Neo) cells, bradykinin (10^{-8} M) inhibits forskolin-stimulated cAMP production by $\sim 50\%$, an effect that is blocked by pertussis toxin. Pertussis toxin alone has no effect on forskolin-stimulated cAMP production. Similar results were obtained with wild-type cells. The cells expressing α_{sQ227L} have higher basal and forskolin-stimulated levels of cAMP production than controls. Bradykinin inhibits forskolin-stimulated cAMP production in these cells by $\sim 20\%$. cAMP production

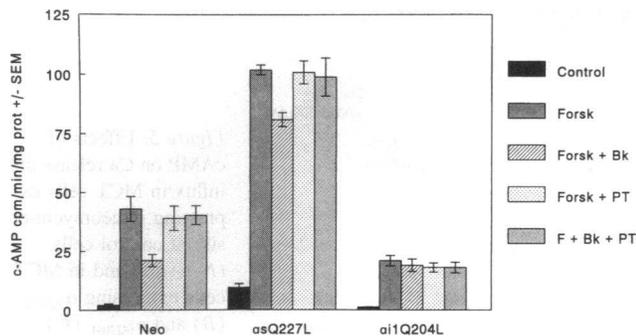


Figure 2. Effect of expression of mutant α chains on hormonal responses in neomycin-resistant MCT cells (*Neo*), MCT cells expressing α_{sQ227L} , and MCT cells expressing $\alpha_{i1Q204L}$. The cells were serum deprived and preincubated with [3 H]adenine overnight. Intracellular cAMP accumulation was measured by separation of [3 H]cAMP from other compounds by column chromatography. Column recoveries were corrected by inclusion of tracer [32 P]cAMP in each sample. Forskolin (100 nM) and bradykinin (10^{-8} M) were added as indicated. Pertussis toxin was present as indicated at 200 ng/ml for 8 h before cAMP production assays. cAMP accumulation is expressed as cpm/min per mg protein \pm SEM. Each point is the mean of triplicate samples, and experiments were repeated three times.

by forskolin-treated cells expressing $\alpha_{i1Q204L}$ is approximately the same level as control cells treated with forskolin and bradykinin. In cells expressing $\alpha_{i1Q204L}$, forskolin increases cAMP production, but less than in control cells, similar to the level found in control cells in the presence of forskolin and bradykinin. Treatment with forskolin and bradykinin, forskolin and pertussis toxin, or forskolin, pertussis toxin, and bradykinin does not significantly alter cAMP production from the forskolin-stimulated level. At the level at which it is expressed in this clonal cell line, $\alpha_{i1Q204L}$ appears to maximally inhibit forskolin-stimulated cAMP accumulation, and bradykinin has no additive effect.

Regulation of intracellular Ca in MCT cells expressing mutant α chains. Bradykinin was used as a potent activator of the phospholipase C–intracellular Ca system (35). A bradykinin dose that produced a maximal effect (10^{-8} M) was chosen based on preliminary dose–response studies. Cells were studied in the absence of CdCl₂ because the effects of both mutant α chains were present under these conditions, and possible complicating effects of Cd were avoided. Hormone-dependent Ca release from intracellular stores in response to bradykinin was studied in a 0 Ca medium to prevent Ca influx across the cell membrane. Ca influx was then measured by adding Ca (2.0 mM) to the medium. Mn-induced quench of intracellular Fura-2 fluorescence was used as a confirmatory measure of plasma membrane Ca permeability because it enters cells by the same pathway as Ca and reflects uptake by IP₃-sensitive stores (15). Expression of the mutant α chains had no significant effect on basal intracellular Ca or bradykinin-induced Ca release from intracellular stores (Fig. 3 A). The basal and peak values (mean \pm SEM) for at least six separate tracings are respectively (Ca nM): 122 \pm 11 and 706 \pm 70 Neo, 99 \pm 13 and 619 \pm 51 α_{sQ227L} , and 106 \pm 9 and 713 \pm 66 $\alpha_{i1Q204L}$. The differences among the peak and basal intracellular Ca values for the three groups were not significantly different ($P > 0.05$, ANOVA). However, expression of these α chains markedly altered Ca influx across the cell membrane in the presence of bradykinin as measured

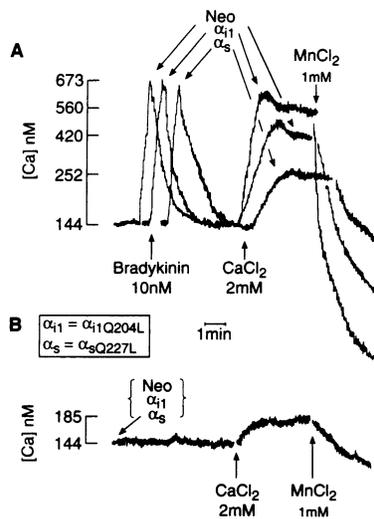


Figure 3. Bradykinin-dependent Ca release from intracellular stores and Ca influx in MCT cells expressing mutant α chains. MCT cells, which had been serum deprived were trypsinized, loaded with Fura-2, and studied in a fluorimeter with constant stirring. In A, release of Ca from intracellular stores in response to bradykinin is compared in control cells (*Neo*) and cells expressing α_{sQ227L} and $\alpha_{i1Q204L}$. Bradykinin (10^{-8} M) was added in Ca-free medium. When intracellular Ca returned to baseline,

CaCl₂ was added to the medium to bring the concentration to 2.0 mM. When intracellular Ca reached a plateau, MnCl₂ was added to a final concentration of 1.0 mM. In B, the experiment was repeated except that bradykinin was not present. The Ca signal could not be calibrated in these individual experiments due to the presence of Mn. However, in similar experiments using aliquots of cells from the same batches and each cell line, release of Ca from intracellular stores was similar in all cell lines and to that shown in A.

by the intracellular Ca plateau and rate of quench of Fura-2 by Mn. Fig. 3 A shows Ca tracings representative of at least six experiments from control neomycin-resistant MCT cells (*Neo*) and cells expressing α_{sQ227L} and $\alpha_{i1Q204L}$. When 2.0 mM Ca is added to the extracellular medium and enters the cells, fluorescence rises in all three cell lines. Ca influx is most rapid (as reflected by the slope of the line) and reaches a higher plateau in cells expressing $\alpha_{i1Q204L}$ and is slowest in cells expressing α_{sQ227L} . A comparison of the intracellular Ca plateau values is provided in Table I along with those for studies with isoproterenol and forskolin (Fig. 4). The rate of Ca influx as reflected by the slope of the tracing after Mn addition is 0.44 ± 0.07 that of control for the α_{sQ227L} -expressing cells, a significant reduction ($P < 0.001$), and 1.52 ± 0.036 for the $\alpha_{i1Q204L}$ -expressing compared to Neo, a significant increase ($P < 0.001$).

As expected on the basis of the Ca studies, expression of the α chains had no effect on IP₃ production [measured with a cerebellar IP₃ binding protein assay (29)]. In Neo cells and cells expressing α_{sQ227L} and $\alpha_{i1Q204L}$, IP₃ production (mean \pm SD) in the basal state was $2.62 \pm 0.287 \times 10^{-9}$, $2.03 \pm 1.46 \times 10^{-9}$, and $2.99 \pm 2.32 \times 10^{-9}$ and rose to a maximum of $3.05 \pm 1.35 \times 10^{-8}$, $2.9 \pm 1.38 \times 10^{-8}$, and $3.12 \pm 1.12 \times 10^{-8}$ at 20 s in the respective cell lines after stimulation with bradykinin. These differences among the basal and peak values are not statistically significant (ANOVA, $P > 0.05$). However, the differences between the peak and basal values were statistically significant ($P < 0.01$, ANOVA).

Ca influx is altered by the presence of mutant α chains only in the presence of bradykinin. Fig. 3 B shows typical tracings from experiments similar to those shown in Fig. 3 A, but without stimulation by bradykinin. In these studies the rate of Ca influx and plateau is similar in all cell lines and substantially less than that in cells treated with bradykinin.

Table I. Comparison of the Plateau Levels of Intracellular Ca in Neomycin-Resistant Control Cells (Neo), and MCT Cells Expressing α_{sQ227L} , and $\alpha_{i1Q204L}$ without and with Isoproterenol (10^{-8} M) or Forskolin 2.5×10^{-5} M

	Control	Isoproterenol	Forskolin
Neo	0.445±0.017	0.333±0.023* <i>P</i> < 0.05	0.26±0.021* <i>P</i> < 0.001
α_{sQ227L}	0.104±0.008 [‡] <i>P</i> < 0.05	0.077±0.015 <i>P</i> > 0.05	0.083±0.088 <i>P</i> > 0.05
$\alpha_{i1Q204L}$	0.63±0.04 [‡] <i>P</i> < 0.001	0.353±0.12* <i>P</i> < 0.001	0.30±0.022* <i>P</i> < 0.001

These studies were performed as described for Figs. 3 and 4. To simplify comparisons, the plateau levels of intracellular Ca are expressed as the fraction of the peak intracellular Ca above the baseline intracellular Ca (mean±SEM). * Values that are statistically different from untreated cells expressing the same α chain. † Values in untreated cells expressing the mutant α chains that statistically different from the Neo control cells by ANOVA.

While the changes in Ca influx could be explained by a direct effect of the α chains on the Ca influx pathway, they could also be due to regulation of Ca influx by the adenylyl cyclase system. To address this issue, we repeated the studies shown in Fig. 3 A in the presence of two activators of the adenylyl cyclase system: isoproterenol, which requires β receptor- α_s -adenylyl cyclase coupling, and forskolin, which acts directly on adenylyl cyclase. Control (Neo) cells and cells expressing α_{sQ227L} and $\alpha_{i1Q204L}$ were pretreated with isoproterenol or forskolin in the presence of IBMX 4 min before the addition of bradykinin (Fig. 4 A, B, and C, respectively). These additions caused no change in basal intracellular Ca or bradykinin-induced Ca release from intracellular stores. However, isoprotere-

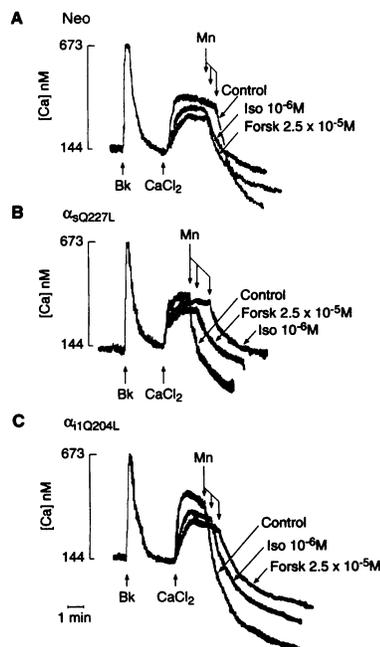


Figure 4. Effects of isoproterenol and forskolin on Ca release and influx in neomycin-resistant control cells (Neo) (A) and in MCT cells expressing α_{sQ227L} (B) and $\alpha_{i1Q204L}$ (C). The cells were serum deprived, trypsinized, loaded with Fura-2, and washed as above. They were then incubated in IBMX (0.2 mM) and isoproterenol (10^{-6} M) or in forskolin (2.5×10^{-5} M) for 4 min before bradykinin (10^{-8} M) was added. The remainder of the experiment was carried out as described in Fig. 3.

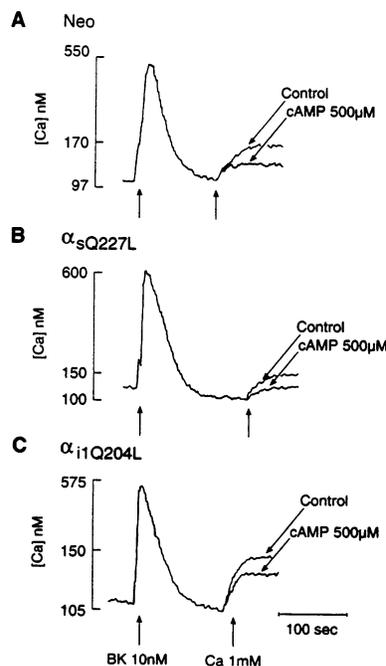


Figure 5. Effects of cAMP on Ca release and influx in MCT cells expressing in neomycin-resistant control cells (Neo) (A) and in MCT cells expressing α_{sQ227L} (B) and $\alpha_{i1Q204L}$ (C). Cells were treated with db-cAMP 15 min (10^{-5} M) before the addition of bradykinin, and Ca was added to a final concentration of 1.0 mM. Otherwise, studies were performed as described in Fig. 3. The tracings were calibrated individually as described in Methods and are typical of at least three experiments.

nol and forskolin reduced Ca influx after bradykinin stimulation. The difference was statistically significant for the Neo and $\alpha_{i1Q204L}$ -expressing cells. The reduction in Ca influx was not significant for the cells expressing α_{sQ227L} , although in paired tracings, differences were evident. Similarly, although paired Ca tracings with isoproterenol and forskolin suggest that forskolin reduces Ca influx to a greater extent than isoproterenol, the differences are not statistically significant. A quantitative comparison of the intracellular Ca plateau level in the three cell lines under control conditions and in the presence of isoproterenol or forskolin is presented in Table I. As in Fig. 3 A, the rate of quench of Fura-2 fluorescence by Mn corresponds to Ca influx and shows decreased rates of quench with forskolin or isoproterenol. These studies suggest that the effects of the expressed α chains on Ca influx are mediated by regulation of the adenylyl cyclase system.

Effects of cAMP on intracellular Ca in MCT cells. To demonstrate that cAMP is required for reduction of Ca influx in MCT cells, MCT cells were preincubated with dibutyl cAMP (db-cAMP) (10^{-4} M) for 15 min before the addition of bradykinin. As shown in Fig. 5 and Table II, cAMP treatment reduced Ca influx in response to the addition of 1 mM extracellular Ca in all three cell lines. The difference was statistically significant for the Neo and $\alpha_{i1Q204L}$ -expressing cells, but not for the cells expressing α_{sQ227L} . Similar results were obtained with chlorophenyl cAMP. These results demonstrate that direct elevation of cell cAMP without activation of receptors, G proteins or adenylyl cyclase is sufficient to alter Ca influx in MCT cells. The fact that Ca influx is not minimal and equal in all cell lines may be due to the relatively low cell permeability of hydrolysis-resistant cAMP analogues.

The role of cAMP-dependent protein kinase in regulation of intracellular Ca in MCT cells. cAMP could regulate Ca influx in MCT cells by direct action such as through a cAMP-gated channel or through PKA. To distinguish between these two possibilities, we treated cells with a chemical inhibitor of

Table II. Comparison of the Plateau Levels of Intracellular Ca in Neomycin-Resistant Control Cells (Neo), and MCT Cells Expressing α_{sQ227L} , $\alpha_{iIQ204L}$ and Rev B

	Control	cAMP	KT-5720
Neo	0.16±0.095	0.088±0.018* <i>P</i> < 0.05	0.19±0.012 <i>P</i> > 0.05
α_{sQ227L}	0.104±0.008 [‡] <i>P</i> < 0.005	0.077±0.015 <i>P</i> > 0.05	0.28±0.038* <i>P</i> < 0.01
$\alpha_{iIQ204L}$	0.248±0.026 [‡] <i>P</i> < 0.005	0.15±0.011* <i>P</i> < 0.05	0.20±0.014 <i>P</i> > 0.05
Rev	0.030±0.055 [‡] <i>P</i> < 0.01	ND	ND

These studies were performed as described in Figs. 5, 6, and 7. To simplify comparisons, the plateau levels of intracellular Ca are expressed as the fraction of the peak intracellular Ca above the baseline intracellular Ca (mean±SEM). * Values that are statistically different from untreated cells expressing the same α chain. ND, not done. [‡] Values in untreated cells expressing the mutant α chains that are statistically different from the Neo control cells by ANOVA.

cAMP-dependent protein kinase, KT-5720 (1 μ M in DMSO for 5 min before Ca studies) and expressed a mutant type I regulatory subunit for PKA that results in cells that are deficient in PKA activity (21, 36). Fig. 6 and Table II show the effects of treatment of the three cell lines with KT-5720. The bradykinin-induced Ca influx is increased in the cells expressing α_{sQ227L} , but not statistically different from untreated control cells expressing $\alpha_{iIQ204L}$. These results indicate that cAMP acts through cAMP-dependent protein kinase to reduce Ca influx in MCT cells. In cells expressing $\alpha_{iIQ204L}$, okadaic acid (10⁻⁷ M) reduced Ca influx to approximately the level seen in untreated Neo cells, but the effects in control (Neo) cells and cells expressing α_{sQ227L}

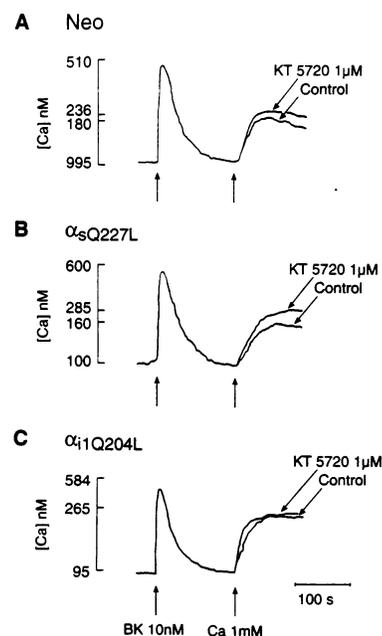


Figure 6. Effects of a chemical inhibitor of cAMP-dependent protein kinase (KT-5720) on Ca release and influx in neomycin-resistant control cells (Neo) (A) and in MCT cells expressing α_{sQ227L} (B) and $\alpha_{iIQ204L}$ (C). The cells were treated with KT-5720 10⁻⁶ M 5 min before the addition of bradykinin and the final concentration of extracellular Ca was 1.0 mM. Otherwise, the studies were performed as described in Figure 3. The tracings were calibrated individually.

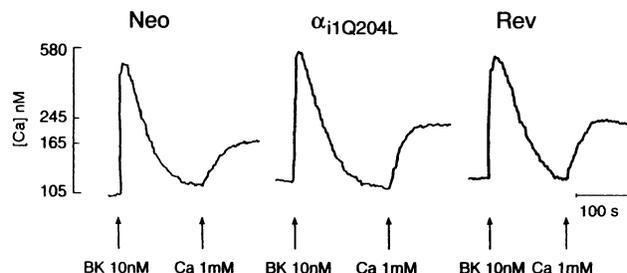


Figure 7. Comparison of the bradykinin-induced Ca signals in neomycin-resistant control cells, cells expressing $\alpha_{iIQ204L}$, and Rev B, a cAMP-resistant PKA regulatory subunit. The studies were performed as described in Fig. 3 except that the final extracellular Ca concentration was 1.0 mM. The tracings were calibrated individually, and are typical of at least five tracings.

were inconsistent. DMSO alone (up to 1%) had no effect on Ca release or influx in these cells.

To confirm the results obtained with KT-5720 with an independent technique, we created clonal cell lines expressing Rev B, a mutant PKA regulatory subunit with reduced affinity for cAMP. Expression of this kinase reduces basal and cAMP-stimulated levels of PKA activity in cells (21). Clonal cell lines expressing Rev B (provided by Stan McKnight, University of Washington) were selected as described above in Methods. The cell line with the highest basal level of Rev B expression based on RNA blots was selected for study because the Ca studies described above were done under basal conditions (in the absence of Cd). Expression of Rev B was further documented by measuring PKA activity in cell extracts. Basal levels of PKA activity (measured as the ability of cell extracts to phosphorylate kemptide, a synthetic peptide substrate for PKA) in cells expressing Rev B and $\alpha_{iIQ204L}$ are reduced compared to control (Neo) cells. The cAMP-stimulated level of kinase activity is reduced only in cells expressing Rev B, whereas basal and forskolin-stimulated cAMP production are similar in control (Neo) cells and cells expressing Rev B. Basal and stimulated (cAMP 10⁻⁵ M) PKA activities (pmol PO₄ transferred/min per μ g cell extract protein) are as follows: 7.2 and 15.7 Neo, 4.6 and 14.7 $\alpha_{iIQ204L}$, and 3.6 and 7.6 Rev B.

Fig. 7 and Table II show a comparison of bradykinin-induced changes in intracellular Ca in control cells, cells expressing $\alpha_{iIQ204L}$, and cells expressing Rev B. The cells expressing $\alpha_{iIQ204L}$ and Rev B are deficient in propagating cAMP-dependent signals. In all three cell types, Ca release from intracellular stores is similar. However, Ca influx across the cell membrane is similar in the cells expressing $\alpha_{iIQ204L}$ and Rev B (ANOVA, *P* > 0.05), but increased compared with controls (ANOVA, *P* < 0.01). These results compliment those obtained with KT-5720 (Fig. 6) and confirm that cAMP regulates bradykinin-induced Ca influx through activation of PKA.

Down-stream signaling effects of α_{sQ227L} and $\alpha_{iIQ204L}$ in MCT cells. To determine if the altered Ca influx pathways in MCT cells expressing mutant α chains have physiologically significant effects on the cells, we studied induction of *c-fos* mRNA by bradykinin. In a number of systems, induction of *c-fos* is partially dependent on extracellular Ca and consequently can serve as a downstream marker for Ca signals (37, 38). In MCT cells ionomycin stimulates *c-fos* expression, so in these cells, a rise in intracellular Ca is sufficient to activate the *c-fos* gene

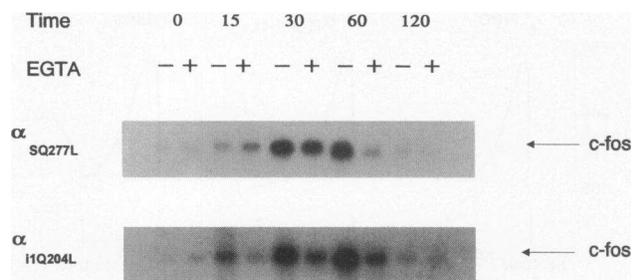


Figure 8. Bradykinin stimulation of *c-fos* expression in cells expressing α_{SQ227L} and α_{HQ204L} . 10-cm dishes of serum-deprived MCT cells were treated with 2 mM EGTA or water 5 min before the addition of bradykinin (10^{-8} M). At the time points indicated, medium was removed and total RNA was prepared. 10 μg of total RNA was size-fractionated on a formaldehyde-agarose gel, transferred to a nylon filter, and hybridized to a probe for *c-fos*. Similar results were obtained in three separate experiments.

(data not shown). Fig. 8 shows the time course of *c-fos* mRNA induction by bradykinin without and with EGTA in MCT cells expressing α_{SQ227L} and α_{HQ204L} . *c-fos* induction by bradykinin is reduced by pretreatment with 1 mM EGTA, indicating that as in other systems, induction of *c-fos* by substances like bradykinin is partially dependent on extracellular Ca. In both cell lines, maximum induction occurs at 60 min, but the induction is greater in the cells expressing α_{HQ204L} , the cell line with the greatest Ca influx after a bradykinin stimulus. Equal amounts of RNA were loaded in each lane, and the loading was verified by ethidium bromide staining of the ribosomal bands and hybridization with a probe for GAPDH. Both blots were processed, hybridized, and exposed at the same time under the same conditions. These studies demonstrate that altered Ca influx at the cell membrane due to increased or decreased adenylyl cyclase can affect signaling at the level of transcription.

Discussion

Expression of mutant α chains. We used mutant, GTPase-deficient forms of α_s and α_i to activate and study signaling pathways that regulate adenylyl cyclase and intracellular Ca in renal epithelial cells. We chose an expression system that would not lead to high level expression of the mutant proteins out of concern for possible loss of specificity. Although significant levels of protein are produced with the metallothionein vector, we expect that they are lower than would have been achieved with viral or retroviral promoter-based expression systems. Loss of specificity due to high levels of protein expression was observed with overexpression of M2 muscarinic receptors in Chinese hamster ovary cells (39, 40). The α chains are active (in the GTP-bound conformation) under the conditions normally found in cells, whereas the endogenous α chains are not (15, 17, 26, 27, 31, 32). The fact that both mutant α chains have a significant spontaneous rate of guanine nucleotide exchange and minimal GTPase activity makes their activation independent of receptor coupling (41, 42). The expression of novel mRNAs and the increase in α_s or α_i antigen over endogenous antigen detected by immunoblotting demonstrate that the cells express proteins with the appropriate biochemical characteristics for constitutively active G protein α chains.

Regulation of adenylyl cyclase by mutant α chains. The

results of these studies indicate that expression of a mutant, activated form of α_i in a renal epithelial cell line inhibits forskolin-sensitive adenylyl cyclase in MCT cells. Expression of a mutant-activated form of α_s stimulates adenylyl cyclase in these cell types. In MCT cells, hormone-sensitive inhibition of adenylyl cyclase is mediated by pertussis toxin substrates (α_{i2} or α_{i3}), but neither expresses α_{i1} .

Although mRNA for types IV, V, and VI adenylyl cyclases, and presumably the corresponding proteins are expressed in whole kidney, their cell-type specific expression in the kidney and renal cell lines is not defined (8, 43–46). Additional forms of adenylyl cyclase may also be identified. Consequently, the form or forms of adenylyl cyclase regulated in these studies are not known at this point. Biochemical studies demonstrate variability among adenylyl cyclase isoforms with respect to regulation by $\beta\gamma$ subunits (5, 7, 8). The present studies demonstrate that adenylyl cyclase isoforms present in MCT cells are regulated in a manner similar to that previously demonstrated in fibroblasts (26, 27, 31, 32).

Inhibition of cAMP accumulation by the normal hormonal mechanisms of these cells and the mutant α_i are not additive indicating that the mechanism of adenylyl cyclase inhibition by α_{HQ204L} and the endogenous mechanism of MCT cells is the same. These results are consistent with those from other studies, including those using purified proteins (26, 27, 47). In the present studies, pertussis toxin treatment of cells blocks bradykinin-induced inhibition of forskolin-stimulated adenylyl cyclase, but has no effect on inhibition of adenylyl cyclase by α_{HQ204L} . This result is expected because pertussis toxin blocks the ability of $\alpha\beta\gamma$ heterotrimers to interact with receptors, but would not be expected to influence receptor-independent guanine nucleotide exchange or effector interactions. Although recombinant, myristoylated α_{HQ204L} is a pertussis toxin substrate in the presence of $\beta\gamma$ subunits in vitro, the efficiency with which it is labeled with respect to the wild-type protein and whether it is a substrate in vivo is not known (Taussig, R., and A. Gilman, personal communication). Consequently, the reason for the lack of an effect of pertussis toxin in the α_{HQ204L} -expressing cells is not defined at this point.

Regulation of intracellular Ca by mutant α chains. In bradykinin-stimulated cells, the mutant α chains affect Ca influx in a manner that correlates inversely with cAMP production. The presence of α_{SQ227L} , which increases cAMP production in MCT cells, reduces Ca influx, whereas the presence of α_{HQ204L} , which reduces cAMP production, increases Ca influx. The studies presented in this manuscript demonstrate that the adenylyl cyclase system acting through PKA is responsible for these observations rather than a direct interaction of α chains or cAMP with the Ca influx pathway. Ca influx in bradykinin-stimulated cells is reduced by stimulation of the adenylyl cyclase system through expression of the mutant activated α_s or the addition of isoproterenol, forskolin, or db-cAMP. Ca influx is increased by reduction of adenylyl cyclase activity through expression of α_{HQ204L} or reduction in PKA activity by treatment with a PKA inhibitor or expression of PKA regulatory subunit with reduced affinity for cAMP. The expressed α chains, isoproterenol, forskolin, cAMP analogues, and inhibition of PKA do not affect bradykinin-induced Ca release from intracellular stores or IP_3 levels.

Ca influx is not altered by the presence of the mutant α chains unless the cells are stimulated by bradykinin (Fig. 3), demonstrating that changes in adenylyl cyclase activity alone are not sufficient to explain these observations. The fact that

stimulation with bradykinin is required to see differences in Ca influx in the three cell lines indicates that the adenylyl cyclase system is regulating the capacitance or refilling Ca influx pathway. The bradykinin-dependent Ca influx pathway in MCT cells is blocked by Ni and La, a characteristic of the "refilling" pathway (15) (Miller, R. T., and K. Kitamura, unpublished observations). Consequently, some component of the signal generated by bradykinin is a prerequisite for alteration of Ca influx by the cAMP pathway. The potential regulators of Ca influx include IP₃, Ca, kinases, NO, cGMP, or calcium influx factor (CIF) (13, 48–52). A recent report shows that in endothelial cells cholera toxin reduces NO-stimulated cGMP production (53). A consequence of this mechanism could be reduced Ca influx after bradykinin stimulation of endothelial cells. These studies suggest that the adenylyl cyclase system is not acting on intracellular Ca release or IP₃ production but may be acting on the generators or targets of these other potential regulators of Ca influx or the influx channel.

The Ca and cAMP signaling systems interact in a number of cell types, the cell type determining the nature of the interaction. In cardiac myocytes, the two systems converge at the Ca channel and act synergistically to increase the inotropic and chronotropic state of the myocardium (10, 11, 54). In contrast, in the collecting duct of the kidney, the actions of vasopressin to stimulate water and Na transport through increases in cAMP are antagonized by substances such as PGE₂, acetylcholine, and bradykinin, which stimulate IP₃ production and raise intracellular Ca (55). However, in the collecting duct, the antagonism does not appear to be at the level of Ca influx. In the present studies, the cAMP system modulates Ca influx in MCT cells after stimulation by agonists that stimulate PI turnover and intracellular Ca release.

Many receptor ligands including bradykinin, acetylcholine, and serotonin simultaneously stimulate IP₃ production with intracellular Ca release and inhibit adenylyl cyclase. Interaction of the adenylyl cyclase and Ca signaling systems such as described in this report could modulate Ca-dependent signals in two ways. In the first, the rapid phospholipase C-dependent rise in intracellular Ca from intracellular stores and the sustained phase of intracellular Ca elevation after phospholipase C activation could be regulated independently. In the presence of bradykinin alone, phospholipase C would be activated and adenylyl cyclase would be inhibited, providing a maximal intracellular Ca signal. Substances that act through α_s would reduce the prolonged intracellular Ca elevation without altering the initial portion of the signal. This scenario is supported by the results obtained with bradykinin-induced *c-fos* expression in MCT cells expressing α_{sQ227L} and $\alpha_{i1Q204L}$.

A second function of the interaction between these two signaling systems could be to modulate the strength of successive Ca-dependent signals. The rate of Ca influx correlates with Ca release from intracellular stores in most cell systems (13, 15). This correlation presumably exists so that signals can be repeated with the same strength. In MCT cells in the presence of activators of adenylyl cyclase, Ca influx is reduced and presumably so is the refilling of intracellular stores. Consequently, to the extent that subsequent signals that depend on Ca release from intracellular stores require refilling of those stores, they will be weaker in the presence of elevated levels of cAMP.

These studies demonstrate that the Ca influx pathway in MCT cells can act as a "signal integrator" for signals generated by the IP₃/intracellular Ca and adenylyl cyclase systems. The

adenylyl cyclase system acting through cAMP-dependent kinase, modulates Ca-dependent signals by altering Ca influx after stimulation by bradykinin. Activation of adenylyl cyclase reduces the sustained phase of the bradykinin-induced Ca signal, whereas inhibition of adenylyl cyclase enhances it. The physiologic effects of this system are demonstrated by the enhanced ability of bradykinin to stimulate *c-fos* expression in the cells with increased bradykinin-induced Ca influx ($\alpha_{i1Q204L}$) and the reduced ability of bradykinin to stimulate *c-fos* expression in cells with reduced bradykinin-induced Ca influx (α_{sQ227L}).

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