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Distinct Calcium Channel Isoforms Mediate Parathyroid Hormone and Chlorothiazide-stimulated Calcium Entry in Transporting Epithelial Cells

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Abstract. Some cells express multiple calcium channel isoforms that are likely to have distinct functions. The present study used molecular cloning and antisense techniques to identify calcium channel isoforms mediating calcium entry in mouse distal convoluted tubule (DCT) cells. The DCT is the major site of hormone- and diuretic-regulated calcium transport in the kidney. Cellular calcium absorption involves entry through apical membrane calcium channels that are sensitive to dihydropyridine-type calcium channel antagonists. Partial cDNA clones corresponding to one isoform of the calcium channel α_1 pore-forming subunit, α_{1C}, and one isoform of the calcium channel β accessory subunit, β3, were isolated by RT-PCR. Full-length transcripts were detected by Northern blot analysis in immortalized DCT cells. Antisense oligonucleotides complementary to the α_{1C} sequence inhibited the rise of intracellular calcium ($[Ca^{2+}]_i$) induced by the thiazide diuretic, chlorothiazide (CTZ), but not that induced by parathyroid hormone (PTH). However, antisense oligonucleotides complementary to the β3 sequence inhibited both CTZ- and PTH-induced rises of $[Ca^{2+}]_i$. β3 antisense oligonucleotides also inhibited the membrane hyperpolarization induced by CTZ but not that triggered by PTH. Thus, members of the voltage-gated calcium channel family are expressed in DCT cells, where they are responsible for hormone- and drug-induced calcium uptake. The results suggest that DCT cells contain multiple calcium channels with distinct roles in the regulation of cellular calcium.

Key words: Parathyroid hormone — Chlorothiazide — cDNA clone — Intracellular Ca^{2+} — Ca^{2+} channels — Ca^{2+} transport

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Introduction

A family of calcium channels has been described in excitable cells that are activated upon membrane depolarization and whose members are distinguished by their unique electrophysiological characteristics and pharmacological sensitivities (Mori et al., 1993; Perez-Reyes & Schneider, 1995). These channels are multimeric complexes, the properties of which are largely conferred by their pore-forming α_1 subunit (Mori et al., 1993; Perez-Reyes & Schneider, 1995). Homologous genes for six types of α_1 subunits have been cloned. These correspond to the different classes of high-voltage activated calcium channels: dihydropyridine-sensitive L-type (α_{1S}, α_{1C}, α_{1D}); ω-conotoxin-GIVA-sensitive N-type (α_{1B}), ω-agatoxin-IVA-sensitive P/Q-type (α_{1A}), and resistant R-type (α_{1E}) channels. The low-voltage activated T-type calcium channel has not been matched to a cloned α_1 subunit. Many cells coexpress multiple $\alpha_1$subunits isoforms (Perez-Reyes et al., 1990; Mori et al., 1993; Mejia-Alvarez, Tomaselli & Marban, 1994; Barry et al., 1995*a;* Schneider et al., 1995). Calcium channels associate with several types of auxiliary subunits (α_2/δ, β, and γ) that may modulate their functional properties (Isom, Jongh & Catterall, 1994; Gurnett, & Campbell, 1996). The most studied and best characterized of these is the β subunit, which is encoded by a family of homologous genes consisting of at least four different isoforms, designated β1–β4 (Castellano et al., 1993*a*). Calcium channels are also expressed in nonexcitable cells where they have important roles in signal transduction and calcium transport. For example, calcium channels with L-type pharmaco-

logical sensitivities have been described in osteoblasts (Karpinski et al., 1989), lymphocytes (Akha et al., 1996), fibroblasts (Soldatov, 1992), pancreatic β cells (Seino et al., 1992), pituitary cells (Cataldi et al., 1996) and kidney epithelial cells (Bacskai et al., 1990).

The kidneys play a central role in calcium homeostasis by regulating calcium absorption from the plasma filtrate (Friedman & Gesek, 1993*a*). In proximal tubules, calcium absorption is primarily passive and not subject to regulation (Friedman & Gesek, 1995*b*). However, in distal convoluted and connecting tubules, active calcium absorption occurs by a transcellular pathway involving calcium influx through apical membrane calcium channels followed by extrusion across basolateral plasma membranes (Bacskai & Friedman, 1990; Matsunaga et al., 1994; Friedman & Gesek, 1995*b;* White, Gesek & Friedman, 1996). Calcium absorption by distal convoluted tubules is regulated by parathyroid hormone (PTH) and calcitonin, and is also stimulated by thiazide diuretics (Gesek & Friedman, 1992*a,b;* Gesek et al., 1993). In isolated tubules or clonal DCT cells, PTH and chlorothiazide, as well as calcitonin and amiloride, cause a sustained rise of $[Ca^{2+}]_i$ that is due to calcium influx and is inhibitable by dihydropyridine-type calcium channel blockers and La^{3+} (Bacskai et al., 1990; Gesek et al., 1992*a;* Poncet et al., 1992; Matsunaga et al., 1994; Friedman & Gesek, 1995*a*). Although the mechanisms and time course of response varies between these agents, in each case calcium entry is associated with membrane hyperpolarization. The molecular identity of the channels responsible for apical calcium entry in DCT cells is unknown.

Multiple isoforms of calcium channel α_1 and β subunits have been detected in kidney (Yu et al., 1992, 1995). Analysis of microdissected nephron segments suggested that different calcium channel isoforms exhibit distinct spatial distributions within the kidney (Yu et al., 1992, 1995). However, the functional roles of the different calcium channel isoforms have not been determined. The goal of the present study was to identify the calcium channel isoforms responsible for calcium entry in DCT cells following stimulation with PTH and the thiazide diuretic chlorothiazide (CTZ). The results reveal that two separate and distinct calcium channels are responsible for calcium influx following treatment with CTZ and PTH.

Materials and Methods

PCR, cDNA, Cloning and Sequencing

The isolation, characterization and culturing of immortalized mouse DCT cells have been described in detail (Pizzonia et al., 1992; Gesek et al., 1992*a,b;* Gesek et al., 1993). Poly (A^+) RNA was isolated from cultured DCT cells using the FastTrack mRNA Isolation Kit (Invitrogen, San Diego, CA) and used for PCR. PCR clones were sequenced by dideoxy sequencing (Sequenase 2.0, United States Biochemical, Cleveland, OH). The products were separated by electrophoresis on 8.0% acrylamide gels (Gel-Mix 8, Gibco BRL). Sequences were determined from both strands of the cDNA and multiple independent clones. Sequence comparisons were performed using online software (Genetics Computer Group, Madison, WI).

α1 Subunit

Reverse transcription and PCR were performed as previously described using conserved oligonucleotide primers [upstream: 5′-GTGG*GAATTC*ATCAAGTCCTTCCAGGCCCT-3′; downstream: 5′-CAGG*GGATCC*AAGTTGTCCATGATGACAGC-3′] (Barry et al., 1995*b*). In brief, reverse transcription, primed with random hexamers, and cDNA amplification were carried out using the GeneAmp RNA PCR kit (Perkin Elmer Cetus, Norwalk, CT) on 250 ng of Poly (A^+). PCR was performed under standard reaction conditions in the presence of 2.0 mM $MgCl_2$ and 0.2 μM of each primer using an annealing temperature of 55°C and 30 cycles. *Bam*H1 or *Eco*R1 restriction sites (underlined) included near the 5′ ends of the primers were used to directionally clone the products of the PCR reaction into the pBluescript II KS-vector (Stratagene, La Jolla, CA). These primers were previously used to amplify α_{1S} and α_{1A} sequences from lung carcinoma cells (Barry et al., 1991) and α_{1C} from UMR-106 osteosarcoma cells (*unpublished results*). PCR amplification of α_1 sequences was also performed as detailed previously (Yu et al., 1992) using two additional sets of primers (Cc1/2 and Cc3/4).

β subunit

Reverse transcription and PCR were performed as previously described using degenerate oligonucleotide primers [B1 (upstream): 5′-AAYAAYGAYTGGTGGATIGG-3′; B2 (downstream): 5′-GCYTTYTGCATCATRTCIGT-3′] (Yu et al., 1995). In brief, 0.2 μg of total RNA was reverse transcribed using SuperScript II reverse transcriptase (Gibco BRL, Gaithersburg, MD) and olido (dT) primers. PCR amplification was with Taq polymerase (Pharmacia Biotech, Piscataway, NJ) in the presence of 2 μM primer and 1.5 mM $MgCl_2$, at an annealing temperature of 55°C. The amplified PCR products were cloned into the *Sma*I site of pBluescript II SK(−).

Northern Blot Analysis

Northern blot analysis of DCT poly (A^+) RNA was performed as previously described using [^{32}P]-labeled probes (Barry et al., 1995*a*). Final high stringency washes were in 0.04 M $NaPO_4$, pH 7.2, 1% SDS, 1 mM EDTA at 65°C. Autoradiography was carried out at −70°C with an intensifying screen. Quantitative analysis of Northern blots was performed by phosphorimage capture (Molecular Dynamics, Sunnyvale, CA) and IPLab Gel software (Signal Analytics, Vienna, VA).

Antisense Analysis

Oligonucleotides were introduced into DCT cells during permeabilization with streptolysin O (Gibco BRL) using a procedure modified from that previously described (Barry, Gesek & Friedman, 1993). Cells in suspension were incubated with 20 U/ml streptolysin O for 20 min at 37°C in the presence of oligonucleotides (sense or antisense). Control cells were permeabilized in the absence of oligonucleotides. Cells were then washed and plated onto glass coverslips. After 6–65 hr, free intracellular calcium, $[Ca^{2+}]_i$, was measured (*see below*). To achieve maximal antisense inhibition for quantitative analysis of β3 mRNA,

Table 1. Antisense oligonucleotide sequences

	Sequence, 5′→3′
α_{1C} Antisense (24-mer)	C C T T C C G T G C T G T T G C T G G G C T C A
α_{1C} Antisense (20-mer)	A C T C T G G G G C A C A C T T C T T G
α_{1S} Antisense	T T C C C A T A G C T G C A G G C C A G
α_{1D} Antisense	A T C T G G T T G T T A T C T C T C A T
α_{1A} Antisense	G G C T T C C C G C T G A G G C A G G
α_{1B} Antisense	C C T T C C C C A C T G T C A T C T C G
α_{1E} Antisense	A G C C A C G A A C A G G T T G A G C A
β3 Antisense (24-mer)	C C C G C C T C C G A G T C C T C A A A C C C G
β3 Antisense (20-mer)	G T C C C A C C A G C A C C A C A G G C

permeabilization was performed 24 hr and again 6 hr before harvesting the cells for isolation of poly (A^+) RNA.

The sequences of the subunit-specific antisense oligonucleotides are given in Table 1. The 20- and 24-mer α_{1C} antisense oligonucleotides and the 20-mer β3 antisense oligonucleotide were designed from the sequences of the DCT cDNA clones described in this work. The other antisense oligonucleotides were designed from published sequences from mouse or rat as follows: β3 24-mer, M88751 (mouse) (Castellano et al., 1993b); α_{1S}, L06234 (mouse) (Chaudhari, 1992); $\alpha_{1D,}$ M57975 (mouse) (Perez-Reyes et al., 1990); α_{1A}, M64373 (rat) (Starr, Prystay & Snutch, 1991); α_{1B}, U04999 (mouse); α_{1E}, L29346 (mouse). Sense oligonucleotide sequences were the reverse complements of their respective antisense oligonucleotide. Oligonucleotides were purchased from Macromolecular Resources (Fort Collins, CO).

Intracellular Calcium and Membrane Voltage

Fluorescent measurements of $[Ca^{2+}]_i$ or membrane voltage were performed using fura-2 AM or 3,3′ dihexyloxacarbocyanine iodide [$DiOC_6(3)$], respectively, as described previously in detail (Gesek & Friedman, 1992*b*). Fluorescent intensity was measured in single cells grown on glass coverslips using a Nikon Photoscan-2.

Statistical Analysis

Comparisons between control and experimental treatment group were evaluated by analysis of variance (ANOVA) and post hoc analysis of multiple comparisons using the Bonferroni method (Instat; Graph-Pad Software, San Diego, CA). Changes of $P \leqslant 0.05$ were assumed to be significant. Curve fitting was performed using a least-squares procedure (Prism, Graph-Pad Software).

Abbreviations

DCT, distal convoluted tubule; $[Ca^{2+}]_i$, free intracellular calcium; CTZ, chlorothiazide; PTH, parathyroid hormone; $DiOC_6(3)$; 3,3′ dihexyloxacarbocyanine; GAPDH, glyceraldehyde phosphate dehydrogenase

Results

Identification of Transcripts Encoding Calcium Channel α_{1C} and β3 Isoforms

Homology-based reverse transcriptase PCR was used to amplify sequences corresponding to calcium channel α_1 and β subunits using primers designed from regions conserved between different isoforms. For the α_1 subunit, partial cDNA clones were isolated corresponding to a single isoform of the calcium channel α_1 subunit and were identified as α_{1C} by sequence comparisons. The 337 bp insert (MDCTA1C) is identical to a published α_{1C} subunit sequence from mouse brain (L01776) (Ma, Holz & Uhler, 1992) and to an overlapping region in a partial sequence from mouse ovary (M57973) (Perez-Reyes et al., 1990). PCR amplification was also performed using two additional sets of primers Cc1/2 and Cc3/4) previously employed to isolate the α_{1S}, α_{1C}, α_{1D}, and α_{1A} isoforms from total rat kidney (Yu et al., 1992). Diagnostic restriction digestion and sequence analysis revealed the presence of only α_{1C} PCR products in DCT cells (*results not shown*).

For the β subunit, PCR amplification resulted in the isolation of partial cDNA clones corresponding to a single isoform, β3. The 259 bp insert (MDCTB3) is 95% identical to a published β3 subunit sequence from rat brain (M88751) (Castellano et al., 1993*b*). There is one conservative amino acid difference between the mouse DCT and rat proteins in this region: at position 39 in the DCT there is a glycine instead of a serine. β3 was the only isoform amplified from DCT cells, whereas β2, β3, and β4 isoforms were isolated from total kidney using the same primers (Yu et al., 1995).

Northern blot analysis was used to detect expression of DCT mRNA transcripts corresponding to the calcium channel clones. A major transcripts of approximately 9.0 kb was detected using the DCT α_{1C} PCR product as a probe (Fig. 1). In addition, a less abundant transcript was present at approximately 12 kb. These are similar in size to transcripts previously reported for α_{1C} in rat brain (Snutch et al., 1991) and aorta (Koch, Ellinor & Schwartz, 1990) and in mouse heart and brain (Ma et al., 1992). Two transcripts, of approximately 3.0 and 2.5 kB, were detected using the DCT β3 PCR product as a probe (Fig. 1). These transcripts are similar in size to those previously reported in rat brain (Castellano et al., 1993*b*). Based on the difference in exposure times, the β3 transcript is more abundant than the α_{1C} transcript in DCT cells.

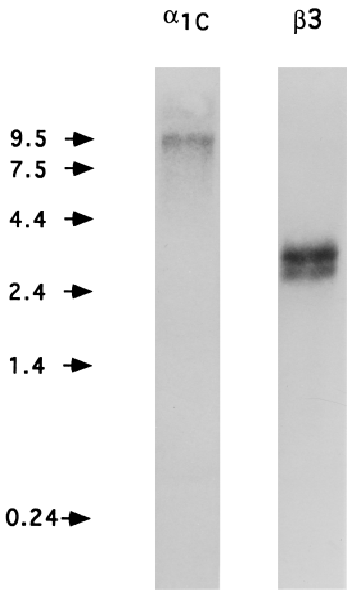

Fig. 1. Northern blot analysis of calcium channel mRNAs in the DCT cell line. Radiolabeled probes synthesized from calcium channel α_{1C} or β3 partial cDNA clones were hybridized to poly (A^+) RNA (10 μg per lane) isolated from DCT cells. Autoradiography was for 115 hr (α_{1C}) or 18 hr (β3). An RNA ladder (Gibco/BRL) was used for size markers (in kilobases).

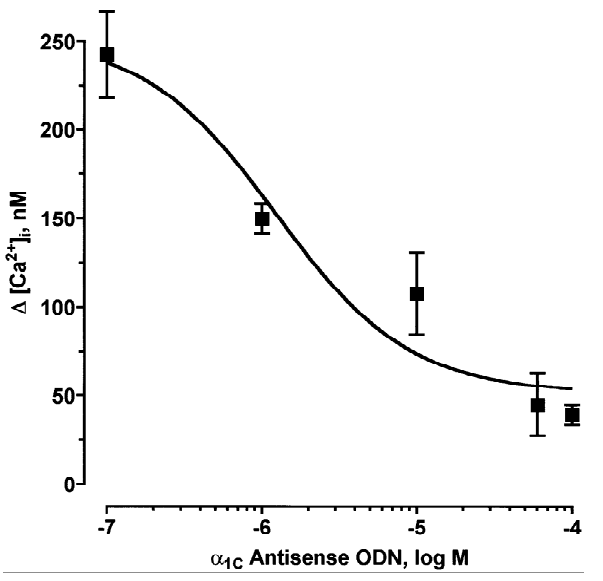

Fig. 2. Concentration dependence of α_{1C} antisense oligonucleotide inhibition of the CTZ-induced rise of $[Ca^{2+}]_i$. DCT cells were treated with increasing concentrations of the α_{1C} 24-mer antisense oligonucleotide. After 18 to 22 hr, the effect of CTZ on $[Ca^{2+}]_i$ was determined. The results are plotted as the difference between the stimulated level of $[Ca^{2+}]_i$ with CTZ (75 μM) and the basal level. Each point denotes the mean ± SE of 3 separate experiments. In permeabilized but untreated controls, the CTZ-induced rise of $[Ca^{2+}]_i$ was 241 ± 13 nM (n = 3).

Inhibition of CTZ-Induced Rise of $[Ca^{2+}]_i$ by Antisense Oligonucleotides Complementary to the α_{1C} Transcript

To examine the functional involvement of the calcium channel α_1 subunit in calcium influx into DCT cells, antisense oligonucleotides were used to inhibit the expression of the α_{1C} isoform. Antisense oligonucleotides to α_{1C} (24-mer) suppressed the CTZ-induced rise of $[Ca^{2+}]_i$ in a dose- (Fig. 2) and time- (Fig. 3) dependent manner. Sense oligonucleotides had no significant effect. Half-maximal inhibition was achieved with 1 μM antisense oligonucleotide (Fig. 2); maximal inhibition occurred at 18 hr (Fig. 3). Incomplete inhibition of the rise of $[Ca^{2+}]_i$ by antisense oligonucleotides is likely due to limitations inherent in the antisense methodology. The maximal level of inhibition achieved is limited by the rate of pre-existing calcium channel turnover and the reduction in the intracellular concentration of oligonucleotide due to degradation by nucleases and cell division (Barry, Gesek & Friedman, 1993).

The ability of the α_{1C} antisense oligonucleotides to inhibit the rise of $[Ca^{2+}]_i$ induced by CTZ and PTH was compared because of the appreciable difference in the kinetics of their effects on calcium transport (Bacskai et al., 1990; Gesek et al., 1992*b;* Friedman & Gesek, 1992; Aubin et al., 1996). These experiments were performed in a paired manner on single cells where the effects of CTZ and PTH were measured in tandem (Fig. 4). Al-

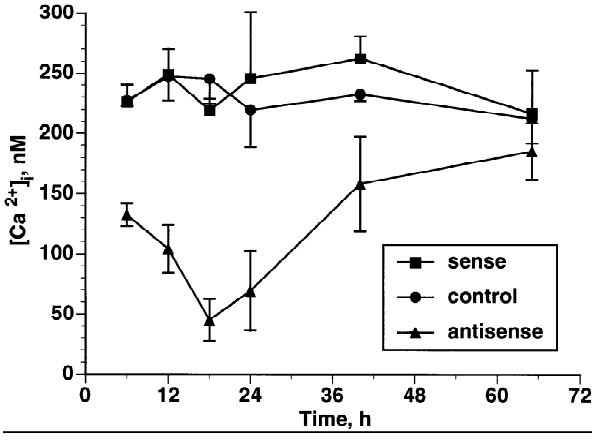

Fig. 3. Time course of α_{1C} antisense oligonucleotide inhibition of the CTZ-induced rise of $[Ca^{2+}]_i$. DCT cells were treated with the α_{1C} 24-mer antisense or sense oligonucleotides (60 μM) or were permeabilized in the absence of oligonucleotide (control). At the indicated times, the cells were assayed for CTZ-induced rises of $[Ca^{2+}]_i$. The results are presented as the difference between the stimulated level of $[Ca^{2+}]_i$ with CTZ (75 μM) and the resting level. Each point denotes the mean ± SE of 3 independent experiments.

though α_{1C} antisense oligonucleotides inhibited the rise of $[Ca^{2+}]_i$ induced by CTZ, they had no effect on the PTH-induced rise of $[Ca^{2+}]_i$ (Fig. 4, Table 2). These results were substantiated by using a second α_{1C} anti-

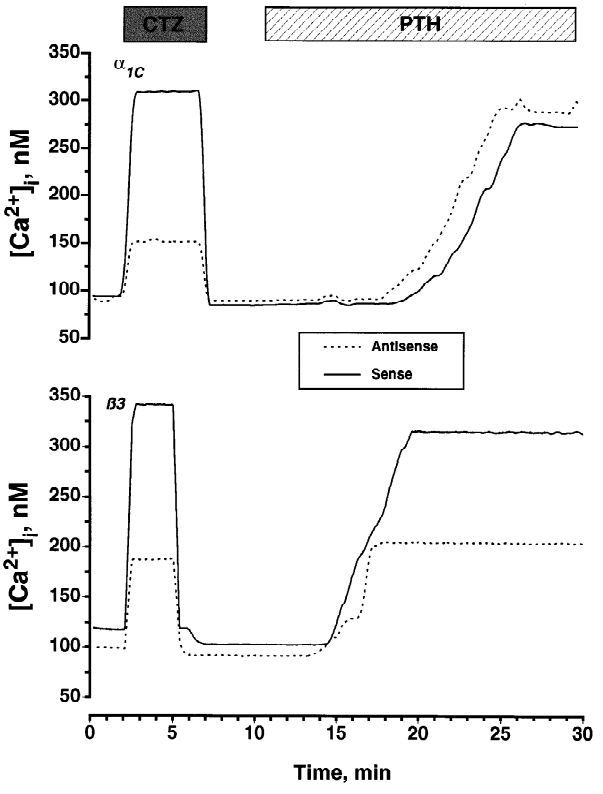

Fig. 4. Effect of α_{1C} (upper panel) and β3 (lower panel) antisense oligonucleotides on PTH- and CTZ-induced rises of $[Ca^{2+}]_i$. Representative traces of $[Ca^{2+}]_i$ in single DCT cells treated with either the α_{1C} 24-mer or β3 20-mer oligonucleotides (100 μM). $[Ca^{2+}]_i$ was measured following addition CTZ (75 μM) or PTH (10 nM) 18 to 24 hr after oligonucleotide treatment. Basal $[Ca^{2+}]_i$ was measured for 2 min, then CTZ was added for 3 or 5 min (lower or upper panel, respectively) and then removed. Basal $[Ca^{2+}]_i$ was again measured for 2 min, then PTH was added for 25 min. Composite results for α_1 and β3 oligonucleotide effects are summarized in Tables 2 and 3, respectively.

Table 2. Summary of effects of calcium channel α_1 subunit oligonucleotides on CTZ and PTH-induced changes of $[Ca^{2+}]_i$.

Treatment	CTZ	PTH
	$\Delta[Ca^{2+}]_i$, % control	
Control	100 ± 4	100 ± 5
α_{1C} 20-mer Antisense	39 ± 5*	96 ± 4
α_{1C} 20-mer Sense	103 ± 1	94 ± 7
α_{1C} 24-mer Antisense	38 ± 10*	96 ± 1
α_{1C} 24-mer Sense	113 ± 4	106 ± 7
α_{1S} Antisense	106 ± 2	102 ± 3
α_{1D} Antisense	102 ± 10	95 ± 8
α_{1A} Antisense	103 ± 4	100 ± 5
α_{1B} Antisense	108 ± 4	98 ± 2
α_{1E} Antisense	106 ± 6	98 ± 4

DCT cells were treated with the indicated calcium channel α_1 subunit oligonucleotides (100 μM). After 18 to 24 hr, paired determinations of $[Ca^{2+}]_i$ in response to CTZ and PTH were made. The difference between the stimulated level of $[Ca^{2+}]_i$ with chlorothiazide (CTZ, 75 μM) or parathyroid hormone (PTH, 10 nM) and the basal level, $\Delta[Ca^{2+}]_i$ was calculated as the percent of that observed in control cells that were not treated with oligonucleotide. Treatment with oligonucleotides had no effect on the basal level of $[Ca^{2+}]_i$. The resting $[Ca^{2+}]_i$ averaged 106 ± 2 nM (n = 34), wheras the CTZ- and PTH-stimulated levels in control cells were 310 ± 7 and 318 ± 11 nM (n = 5), respectively. Values are the means ± SE of 3 to 5 independent experiments. *P < 0.001 (*vs.* control).

sense oligonucleotide (20-mer) that was designed to a sequence just upstream of the 24-mer α_{1C} antisense oligonucleotide. The 20-mer α_{1C} antisense oligonucleotide effectively inhibited the CTZ-induced rise of $[Ca^{2+}]_i$, but had no effect on the PTH-induced rise of $[Ca^{2+}]_i$ (Table 2).

In an attempt to identify the α_1 isoform responsible for PTH-induced calcium influx, antisense oligonucleotides were designed from each of the published sequences of other α_1 subunit isoforms, α_{1S}, α_{1D}, and α_{1A}, which are expressed in kidney (Yu et al., 1992), and α_{1B} and α_{1E}, which have not been reported in kidney. These oligonucleotides were tested for their ability to inhibit induced rises of $[Ca^{2+}]_i$ despite the fact that PCR products corresponding to these isoforms were not detected in DCT cells. None of these α_1 subunit antisense oligonucleotides affected CTZ- or PTH-induced rises of $[Ca^{2+}]_i$ (Table 2). Thus, although the present results implicate the calcium channel α_{1C} isoform in CTZ-induced calcium entry, the α_1 isoform responsible for PTH-induced calcium entry in DCT cells remains unknown.

Inhibition of CTZ- and PTH-Induced Rises of $[Ca^{2+}]_i$ by Antisense Oligonucleotides Complementary to the β3 Transcript

The involvement of the calcium channel β subunit in calcium influx was examined using antisense oligonucleotides to inhibit the expression of the β3 isoform cloned from DCT cells. Antisense oligonucleotides (20-mer) to the β3 isoform inhibited the rises of $[Ca^{2+}]_i$ elicited by PTH and CTZ, whereas the corresponding sense oligonucleotides had no effect (Fig. 4, Table 3). When a second, nonoverlapping, β3 antisense oligonucleotide (24-mer) was used, similar results were obtained (Table 3). These findings suggest that the β3 subunit is a common component of the calcium channels involved in CTZ- and PTH-induced calcium entry in DCT cells.

Treatment of DCT cells with either α_{1C} or β3 antisense oligonucleotides caused only partial inhibition of the rise of $[Ca^{2+}]_i$ induced by CTZ (Tables 2, 3). Therefore, a combination of both antisense oligonucleotides was used to determine whether their inhibitory effects are additive. In permeabilized but untreated control cells the rise of $[Ca^{2+}]_i$ was 214 ± 13 nM (n = 3; 100 ± 6%).

Table 3. Summary of the effects of calcium channel β3 subunit oligonucleotides on CTZ and PTH-induced changes of $[Ca^{2+}]_i$

Treatment	CTZ	PTH
	$\Delta[Ca^{2+}]_i$, % control	
Control	100 ± 7	100 ± 8
β3 20-mer Antisense	46 ± 3*	56 ± 3*
β3 20-mer Sense	95 ± 2	95 ± 3
β3 24-mer Antisense	36 ± 5*	40 ± 6*
β3 24-mer Sense	79 ± 6	84 ± 6

DCT cells were treated with the indicated calcium channel β3 subunit oligonucleotides (100 μM). $[Ca^{2+}]_i$ was measured after 18 to 24 hr as described in the legend to Table 2. Resting $[Ca^{2+}]_i$ averaged 103 ± 2 nM (n = 22); CTZ- and PTH-stimulated $[Ca^{2+}]_i$ in control cells were 343 ± 17 and 323 ± 18 nM (n = 6), respectively. Treatment with oligonucleotides had no effect on the basal level of $[Ca^{2+}]_i$. Values are the means ± SE of 4 to 6 separate experiments.
*P < 0.001 (*vs.* control).

Table 4. Effect of β3 subunit oligonucleotides on membrane voltage

Treatment	CTZ	PTH
	Δ Ve, mV	
Control	−16.1 ± 0.4	−18.5 ± 1.1
β3 Antisense	−7.3 ± 0.4*	−20.2 ± 2.4
β3 Sense	−17.4 ± 2.1	−17.4 ± 0.9

DCT cells were treated with the indicated calcium channel β3 subunit oligonucleotides (20-mer, 100 μM) or permeabilized in the absence of oligonucleotide (control). After 18 to 24 hr, the effect of chlorothiazide (CTZ, 75 μM) or parathyroid hormone (PTH, 10 nM) on membrane voltage (Δ Ve) was measured as described in Materials and Methods. Values are the mean ± SE of 3 experiments. Changes of membrane voltage were determined by a null-point calibration with graded concentrations of external K^+ in the presence of 10^{-6} M valinomycin (Gesek et al., 1992*b*). The changes of membrane voltage were (n = 9): 1 mM $[K^+]$: −37.9 ± 0.9 mV; 3 mM $[K^+]$: −17.3 ± 2.3 mV; 5 mM $[K^+]$: −0.0 ± 1.7 mV; 10 mM $[K^+]$: +36.7 ± 5.5 mV.
*P < 0.05 (*vs.* control).

The increase of $[Ca^{2+}]_i$ measured in cells treated with both α_{1C} and β3 antisense oligonucleotides (41 ± 1% of control) was identical to that obtained in cells treated with either the α_{1C} antisense (42 ± 4% of control) or the β3 antisense (44 ± 7% of control) oligonucleotides alone. These results are consistent with the view that α_{1C} and β3 subunits are components of a single calcium channel complex that is responsible for calcium influx in response to CTZ.

Antisense Oligonucleotides Complementary to the β3 Transcript Inhibit CTZ—But not PTH-Induced Membrane Hyperpolarization

Previous studies of the mechanism of PTH stimulation of calcium transport in DCT cells revealed that membrane hyperpolarization occurred first or contemporaneously with a rise of $[Ca^{2+}]_i$ (Gesek & Friedman, 1992*a*). Notably, membrane hyperpolarization was independent of calcium influx since it was not inhibited by the dihydropyridine calcium channel blocker nifedipine, whereas manipulations that inhibited hyperpolarization also decreased calcium influx (Gesek & Friedman, 1992*a*). Thus, it might be anticipated that antisense oligonucleotides to the calcium channel would not inhibit PTH-induced membrane hyperpolarization. This hypothesis was tested using antisense oligonucleotides to the β3 subunit (Table 4). In DCT cells treated with β3 antisense oligonucleotides, the magnitude of membrane hyperpolarization induced by PTH was not different from that measured in control cells or cells treated with β3 sense oligonucleotides. However, the magnitude of membrane hyperpolarization induced by CTZ in cells treated with β3 antisense oligonucleotides was reduced to 45% of that in control cells and in cells treated with β3 sense oligonucleotides. Consistent with these data, antisense oligonucleotides to the calcium channel α_{1C} subunit also reduced the CTZ-induced hyperpolarization to a similar extent as the β3 antisense oligonucleotides, while α_{1C} sense oligonucleotides had no effect (*data not shown*).

Antisense Oligonucleotides Complementary to the β3 Transcript do not Suppress β3 mRNA Levels

Antisense oligonucleotides to calcium channel transcripts are presumed to reduce the rise of $[Ca^{2+}]_i$ measured in DCT cells by specifically inhibiting the expression of the calcium channel protein. Inhibition of protein expression may occur directly as a result of oligonucleotide interference with mRNA translation, or may result from the specific reduction in mRNA levels by RNase H-mediated degradation (Wagner, 1994). This later possibility was tested for the β3 mRNA transcript using quantitative Northern blot analysis. The mRNA for glyceraldehyde phosphate dehydrogenase (GAPDH) served as an internal standard to normalize the samples for RNA content. In three experiments, the level of β3 mRNA was not significantly different in cells treated with antisense (98 ± 23%) or sense (99 ± 9%) oligonucleotides compared to permeabilized but untreated control cells (100 ± 6%). These results suggest that antisense inhibition occurs at the level of β3 mRNA translation.

Discussion

Previous studies demonstrating that calcium entry into DCT cells is enhanced by PTH (Bacskai et al., 1990;

Gesek & Friedman, 1992*a;* Friedman et al., 1996) and by thiazide diuretics (Gesek & Friedman, 1992*b;* Friedman & Gesek, 1993*b*) are consistent with the well established physiological and pharmacological regulation of calcium absorption by this nephron segment (Friedman & Gesek, 1995*b*). Calcium entry in DCT cells is inhibited by dihydropyridine-type calcium channel blockers and verapamil (Bacskai et al., 1990; Poncet, Merot & Poujeol, 1992; Gesek & Friedman, 1992*a;* Matsunaga et al., 1994), implicating L-type calcium channels. The sensitivity to dihydropyridine-type drugs, notwithstanding, the functional properties of the DCT calcium entry channel differ from L-type calcium channels in a number of respects. The DCT calcium channel is characterized by a low single-channel conductance (2-15 pS), prolonged open times, and an open channel probability that increased at hyperpolarizing voltages (Poncet et al., 1992; Tan & Lau, 1993; Matsunaga et al., 1994). A calcium entry channel with a somewhat different electrophysiological profile, but that is activated upon membrane hyperpolarization, was reported in connecting tubules (Tan & Lau, 1993). Although the molecular identity of the DCT channel is not known, two general possibilities regarding its structure can be envisioned. First, the channel may be structurally distinct from previously isolated calcium channels and therefore represent a new class of calcium channels. Second, the DCT calcium channel could be a member of the known voltage-gated calcium channel family. In this case, the unique properties of the channel may be conferred either by alternative splicing of previously isolated members of the family, most likely one of the L-type α_1 subunits, or it might be composed of a novel α_1 subunit that has not been isolated previously. The results discussed below are consistent with this latter alternative.

The goal of the present investigation was to determine the molecular identity of the channel(s) responsible for calcium entry into transporting DCT cells following stimulation with PTH and the thiazide diuretic, CTZ. A two-step approach was employed. In the first, RT-PCR was used to isolate partial cDNAs for the expressed calcium channel subunits, and in the second, antisense techniques were applied to determine the functional involvement of these expressed channels in mediating calcium influx in response to PTH and CTZ.

cDNA clones corresponding to calcium channel α_1 and β subunits were isolated from DCT cells. Only the α_{1C} and $\beta 3$ isoforms were found; their presence was confirmed by Northern blot analysis. The PCR primers used in this study have been employed previously to amplify multiple isoforms of α_1 and β subunits from different tissues (Barry & Froehner, 1991; Yu et al., 1992, 1995). Their failure to do so in DCT cells suggests that other isoforms are either not expressed or are relatively rare.

Consistent with the results reported here, earlier work by Yu and Lytton and coworkers localized mRNAs for the α_{1C} (Yu et al., 1992) and $\beta 3$ (Yu et al., 1995) calcium channel subunit isoforms to the kidney cortex by Northern blot analysis of macroscopically dissected kidney. However, only α_{1A} and $\beta 4$ were localized to the distal tubule by RT-PCR analysis of individual nephron segments (Yu et al., 1992, 1995). These observations led the authors to speculate that α_{1A} and $\beta 4$ are components of the apical calcium uptake channel in DCT cells. However, the α_{1A} isoform is insensitive to dihydropyridines (Mori et al., 1991), contrary to what would be expected for the DCT calcium channel. In addition, it is possible that PCR of individual segments is not sufficiently sensitive to detect other α_1 and β isoforms expressed because of the limited quantity of RNA isolated. In fact, using this technique, expression of calcium channel α_1 subunits was not detected in any other nephron segment (Yu et al., 1992; Yu, 1995). Furthermore, RNA isolated from microdissected distal tubule nephron segments may originate from multiple cell types found in that region or from contaminating adherent cells. Thus, the use of the clonal DCT cell line in the present work has the advantage of providing a homogeneous source of relatively unlimited amounts of RNA.

In the second part of this study, antisense oligonucleotides specific for the calcium channel α_{1C} and $\beta 3$ isoforms were used to determine their involvement in calcium influx. In previous work, the rise of $[Ca^{2+}]_i$ due to PTH (Bacskai et al., 1990; Gesek & Friedman, 1992*a*) or CTZ (Gesek & Friedman, 1992*b*) was completely blocked by the dihydropyridine antagonist nifedipine or by removal of extracellular calcium indicating that it is due solely to calcium entry through dihydropyridine-sensitive channels. Antisense oligonucleotides to both α_{1C} and $\beta 3$ inhibited the CTZ-induced rise of $[Ca^{2+}]_i$ to a similar extent. The effects of the two distinct antisense oligonucleotides were not additive, consistent with their action on a single calcium channel that is composed of both $\alpha_{1C}$and $\beta 3$ subunits. However, when DCT cells were treated with PTH, only $\beta 3$ antisense oligonucleotides inhibited the rise of $[Ca^{2+}]_i$, while α_{1C} antisense oligonucleotides had no effect. Because $\beta 3$ antisense oligonucleotides were effective inhibitors, these results support the hypothesis that the PTH-activated calcium channel is a member of the extant voltage-gated, multimeric calcium channel family. However, the identity of the α_1 subunit remains to be determined. Antisense oligonucleotides to the other α_1 isoforms failed to inhibit the PTH-induced rise of $[Ca^{2+}]_i$. Thus, it seems likely that another, as yet unidentified, member of the calcium channel α_1 subunit family is responsible for PTH-induced calcium influx. The present results indicate that the $\beta 3$ subunit is shared by two distinct α_1 subunits in DCT cells. Promiscuous coupling between α_1 and β

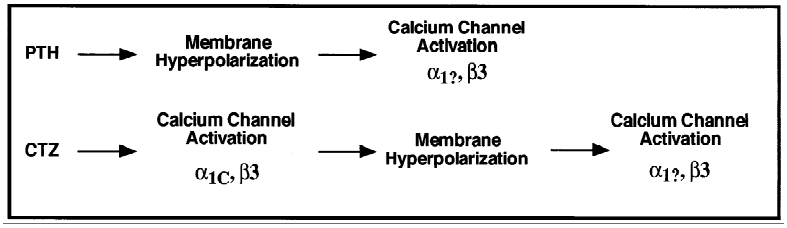

Fig. 5. Model for multiple calcium entry channels. This working model summarizes some components of the proposed pathways of calcium entry in DCT cells. Following treatment with PTH, membrane hyperpolarization leads to calcium influx through a channel comprised of a β3 subunit and an unknown α_1 subunit ($\alpha_{1?}$). A different channel complex, composed of α_{1C} and β3 subunits, is activated by CTZ. Activation of the α_{1C} calcium channel precedes hyperpolarization, which in turn activates the PTH-sensitive channel as well.

subunit isoforms has been reported in studies of native cells and in heterologous expression systems (Scott et al., 1996; Liu et al., 1996).

For studies employing antisense strategies it is important to include controls that demonstrate the specificity of the effects (Wagner, 1994). In the present work, β3 mRNA levels were measured because their relative abundance makes quantification feasible following large scale antisense treatment of DCT cells. However, there was no measurable reduction in β3 mRNA levels in cells treated with antisense oligonucleotides suggesting that the inhibition occurs at the level of protein translation. Furthermore, data from the functional analyses provides convincing evidence for the specificity of the antisense effects of α_{1C} and β3 oligonucleotides. For both the α_{1C} and β3 subunits identical results were obtained with two sets of independent (nonoverlapping) oligonucleotides (20- and 24-mers). In addition, for α_{1C} antisense oligonucleotides, only the CTZ- and not the PTH-induced rise of $[Ca^{2+}]_i$ was inhibited, indicating that cellular function was not generally impaired. Likewise, for β3 antisense oligonucleotides, only the CTZ- and not the PTH-induced membrane hyperpolarization was inhibited. In both cases, the selectivity of the effect of the α_{1C} or β3 antisense oligonucleotides on either CTZ or PTH-mediated events demonstrates that the inhibition observed does not result from a nonspecific suppression of cellular activity.

The present observation demonstrate that distinct calcium channels are activated following treatment of DCT cells with CTZ or PTH. These findings are consistent with the findings of Lajeunesse et al., who suggested that PTH and hydrochlorothiazide increased calcium transport by acting through distinct calcium transport pathways (Lajeunesse, Bouhtiauy & Brunette, 1994). In their study, calcium uptake was measured in luminal membrane vesicles isolated from distal nephron segments. PTH affected a high affinity component of calcium uptake, whereas the effect of hydrochlorothiazide was on a low affinity component. PTH and CTZ exhibit strikingly different kinetics on calcium entry in DCT cells (Gesek & Friedman, 1992*a*, *b*). Stimulation of calcium influx by CTZ occurs within seconds, while that elicted by PTH requires several minutes and is dependent upon protein synthesis (Gesek & Friedman, 1993). Further evidence for differences between the mechanisms of PTH- and CTZ-induced calcium influx was found in the present study from experiments utilizing β3 antisense oligonucleotides. Although both CTZ- and PTH-induced calcium influx is associated with membrane hyperpolarization, β3 antisense oligonucleotides had no effect on the PTH-induced hyperpolarization, while they reduced the hyperpolarization in response to CTZ. These results are compatible with previous studies suggesting that PTH hyperpolarizes membrane voltage, which in turn leads to calcium influx through an apical membrane calcium entry channel (Bacskai & Friedman, 1990; Gesek & Friedman, 1992a; Matsunaga et al., 1994). However, they suggest that in the case of CTZ the pathway leading to hyperpolarization involves the activation of a distinct calcium channel. Calcium channels with unusual voltage-gating properties are also expressed in other types of nonexcitable cells and phosphorylation is likely to play a role in their regulation. For example, a voltage-insensitive L-type calcium channel is expressed in B lymphocytes and is regulated by cGMP (Akha et al., 1996).

The implications of the present study are summarized schematically in Fig. 5. According to the proposed model, PTH treatment leads to membrane hyperpolarization, which in turn stimulates calcium influx through a channel composed of an unknown α_1 subunit ($\alpha_{1?}$) and a β3 subunit. This PTH-sensitive channel is thought to represent the apical membrane calcium entry channel that mediates calcium absorption in response to physiological stimulation of the distal nephron. In contrast, CTZ treatment first causes calcium influx through a channel composed of α_{1C} and β3 subunits, and subsequently leads to hyperpolarization and calcium influx through the PTH-sensitive channel. Involvement of the later calcium channel is speculative and can not be tested without identification of the $\alpha_{1?}$ subunit. The mechanism responsible for activation of the α_{1C}/β3 channel is not known, but may involve its sensitivity to volume changes following treatment with relatively high doses of CTZ. Evidence for activation of a calcium channel α_{1C} isoform during cell swelling was previously reported in a osteosarcoma cell line (Duncan et al., 1996). Thus,

the CTZ- and PTH-sensitive calcium channels expressed in DCT cells are likely to have distinct roles in the regulation of cellular calcium.

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