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Calcium Increases Apolipoprotein B mRNA Editing

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ApoB-100 and apoB-48 are major components of chylomicrons, very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL). The two proteins are generated from a single apoB mRNA by apoB mRNA editing which induces an in-frame stop codon in apoB mRNA. Apolipoprotein B (apoB) mRNA editing is an important determinant of the proportion of full-length (apoB-100) and truncated (apoB-48) proteins in total apoB metabolism. Calcium is involved in the regulation of secretion and synthesis of VLDL and apoB. In this paper, we demonstrate for the first time that the amount of edited apoB mRNA in the cultured cells Caco-2 and McA7777 is markedly increased by calcium. Increasing extracellular calcium concentration, calcium ionophore (A23187 and ionomycin) treatment, and depleting calcium stores and raising cytoplasmic calcium concentration by thapsigargin increase apoB mRNA editing up to threefold in a dose dependent manner. Calcium has no direct stimulative effect on apoB mRNA editing in an *in vitro* editing system. The editing increase by extracellular calcium is not related to alterations of APOBEC-1 mRNA expression. These data suggest that calcium is not only involved in the regulation of apolipoprotein metabolism but also apoB mRNA editing.

Posttranscriptional apoB mRNA editing generates two apoB mRNA molecules with a single nucleotide alteration by a site-specific deamination reaction which converts a single C to U in the glutamine codon (CAA) at amino acid position 2153 to an in-frame stop codon (UAA), resulting in the translation of a truncated apoB-48 as well as full length apoB-100 (1, 2). The amount of edited apoB mRNA is an important determinant of the proportion of full-length (apoB-100) and truncated (apoB-48) proteins in total apoB (3–5), LDL and VLDL metabolism (6). ApoB mRNA editing is catalyzed by a multiple protein enzyme complex which is incompletely characterized. Current data show that it is composed of a catalytic subunit designated APOBEC-1 (7), APOBEC-1 Complementation Factor (ACF) (8, 9), and other incompletely identified auxiliary proteins (9–11). APOBEC-1 alone cannot catalyze the editing of apoB mRNA *in vitro* in the absence of auxiliary proteins (10, 12, 13). ApoB mRNA editing is under tissue specific, developmental and metabolic regulation *in vivo.* Many factors have been reported to be able to regulate the apoB mRNA editing. These include a variety of dietary and hormonal interventions: ethanol treatment (14, 15), thyroid hormone treatment (16), fasting and carbohydrate refeeding (5), high dose estrogen treatment (17), growth hormone (18), and chronic insulin treatment (19). Recently it has been reported that the enzyme activity of apoB mRNA editing is present in both nuclei and cytoplasm and that the nuclear apoB mRNA editing increase has a rapid onset with ethanol treatment (15). It has also been found that there are inhibitory components, hnRNP C1 protein and 40S hnRNP complexes which co-exist with the apoB mRNA editing enzyme complex (20). Despite much investigation, the molecular mechanisms regulating apoB mRNA editing remains largely unknown.

Apolipoprotein B is the major structural protein of chylomicrons, very low density lipoproteins (VLDL) and low density lipoprotein (LDL) particles and plays a fundamental role in determining the metabolic fate of ingested and *de novo* synthesized triglycerides and cholesterol (6, 21). ApoB is also a calcium binding protein (22). LDL transiently increases cytoplasmic calcium concentration (23). Calcium has been shown to be involved in the regulation of VLDL-associated apoB protein synthesis and secretion. Disruption of cellular calcium homeostasis with a calcium ionophore (ionomycin or A23187) results in dose-specific increases in apoB synthesis and secretion in Caco-2 cells (24). A23187 and the calcium antagonists verapamil and diltiazem inhibit VLDL-associated apoB synthesis and secretion

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in primary cultures of rat hepatocytes (25–27). The calcium antagonist AE0047 suppresses the secretion of apoB in human intestinal cell line Caco-2 but increases the cellular uptake of VLDL in human hepatoblastoma Hep G2 cells (28). VLDL secretion reaches a maximum in rat primary hepatocytes cultured in medium containing 0.8–2.4 mM calcium. Either lower or higher calcium concentration or treating the cells with the Ca^{2+}-ATPase inhibitor thapsigargin suppresses lipoprotein secretion (29).

In this present study, we examined the effect of varying cellular calcium levels on apoB mRNA editing in human intestinal cell line Caco-2 and rat hepatoma cell line McA7777. Our data demonstrate that calcium can increase apoB mRNA editing, suggesting that calcium is not only a mediator of apolipoprotein B secretion but also of apoB mRNA editing.

MATERIALS AND METHODS

Cell culture and treatment. The human colon adenocarcinoma cell line Caco-2 and rat hepatoma cell line McA7777 were purchased from ATCC (Rockville, Maryland). Caco-2 cells were maintained in DMEM containing 10% fetal bovine serum. McA7777 cells were maintained in DMEM containing 20% horse serum and 5% fetal bovine serum on plates pre-coated with collagen (Roche). Caco-2 cells were cultured at 37°C for 14 days before treatment. The agents for the cell treatment were dissolved in DMSO and were added to culture medium to a concentration indicated. The final concentration of DMSO was less than 0.05% and the control was performed with 0.05% DMSO. After treatment, total cellular RNA was isolated by Trizol reagent (GIBCO BRL) following the manufacturer's instruction.

ApoB mRNA editing assay. The endogenous apoB mRNA editing in cells was determined by primer extension following RT-PCR amplification as previously described (30). Total cellular RNA samples were pre-treated with DNase RQ1 (Worthington) and Sau 3AI (GIBCO BRL). The first strand cDNA was generated with a random primer using M-MLV reverse transcriptase and the PCR was performed for the amplification of apoB. The PCR conditions were as the follows: for human apoB, 35 cycles of 1 min at 94°C, 1 min at 58°C, 2 min at 72°C; for rat apoB, 5 cycles of 1 min at 94°C, 1 min at 60°C, 2 min at 72°C followed by 32 cycles of 1 min at 94°C, 1 min at 56°C, 2 min at 72°C. The RT-PCR amplicon of 415 or 297 bp for human or rat apoB flanking the edited base were then purified using a GeneClean II kit (Bio 101) and annealed to an antisense ^{32}P-labeled oligonucleotide (apoB-extension-primer) at 42°C for 1 h. Primer extension was performed at 42°C for 10 min as previously described (30). The primer extension products were then resolved on an 8% polyacrylamide–urea gel and the ratio of edited to unedited apoB mRNA was determined by a PhosphoImager.

In vitro apoB RNA editing assay. The *in vitro* apoB RNA editing assay was performed by incubating 10 fmol of a synthetic 419 nt human apoB RNA (nucleotide position 6471-6889) with 15 μg rat liver protein extract at 30°C for 3 h in 20 mM HEPES, pH 7.9, 50 mM KCl, 10 mM EDTA, 1 mM DTT, 10% glycerol, and 40 units of RNase inhibitor in a final volume of 60 μl as previously described (31). Following the reaction, the RNA was extracted and subjected to RT-PCR and primer extension as described above to determine the proportion of edited and unedited apoB mRNA.

Quantitation of mRNA abundance by competitive RT-PCR. Quantitative RT-PCR was conducted using a competitor DNA generated by internal deletion as an internal standard as previously described (32). Three micrograms of total cellular RNA of Caco-2 cells were taken for each sample and the first strand cDNA was generated with a random hexamer primer using M-MLV reverse transcriptase. cDNA aliquots with the same volume were amplified by PCR for beta-2-microglobulin (β2M), APOBEC-1 and apoB in the presence of a selected concentration of competitor and 0.3 μl [α-^{32}P]dCTP (3000 Ci/mmol, 10 Ci/ml, ICN). PCR amplification was performed in a RoboCycler Gradient-40 (Stratagene) as follows: (a) β2M, 2 min at 94°C, 21 cycles of 30 s at 94°C, 1 min at 60°C, 2 min at 72°C, and 10 min at 72°C; (b) APOBEC-1, 2 min at 94°C, 22 cycles of 30 s at 94°C, 1 min at 60°C, 2 min at 72°C, and 10 min at 72°C; and (c) apoB, 2 min at 94°C, 20 cycles of 30 s at 94°C, 1 min at 58°C, 2 min at 72°C, and 10 min at 72°C.

The DNA competitors utilized above were generated by a PCR amplification of target cDNA using the target DNA sense primer and an antisense primer located near 50 nt upstream from the target DNA antisense primer site and tailed with the target DNA antisense primer as previously described (32). The resultant PCR products (10 μl aliquot) were resolved by 4% polyacrylamide gel electrophoresis and quantified by a PhosphoImager. The ratio of target to competitor product represented the mRNA relative abundance in multiple samples. The quantitation of β2M mRNA served as an internal standard for the estimation of the starting mRNA level and the APOBEC-1 or apoB mRNA abundance was normalized according to β2M levels by dividing the ratio of APOBEC-1 mRNA with corresponding ratio of β2M and multiplying with the ratio mean value of β2M in control group.

Oligonucleotide. Human apoB-S, 5′-CTGGGAAAACTCCCACAG-CAAG-3′; human apoB-AS, 5′-CCACATTTTGAATCCAGGATGCAG-3′; human apoB-extension-primer, 5′-TATCTTTAATATACTGATC-3′; rat apoB-S, 5′-AGAGGATCCCTGAGCAGGCTTCCTCAGCAG-3′; rat apoB-AS, 5′-TTAAAGCTTCAATGATTCTATCAATAATCTG-3′; rat apoB-extension-primer, 5′-TATCTCTAATATACTGATC-3′; rat β2M-S, 5′-AAGCCCAACTTCCTCAACTGCTAC-3′; rat β2M-AS, 5′-GATGATTCAGAGCTCCATAGAGCTTG-3′; β2M-competitor-AS, 5′-GATGATTCAGAGCTCCATAGAGCTTGTTTAACTCTGCAAG-3′; rat APOBEC-1-S, 5′-CCCCGGGAACTTCGGAAAGAG-3′; rat APOBEC-1-AS, 5′-GGGGGTACCTTGGCCAATG-3′; and APOBEC-1-competitor-As, 5′-GGGTACCTTGGCCAATGAGCTTGCTCCGTCATGATCTGG-3′.

RESULTS

Calcium increases apoB mRNA editing. To examine the effect of calcium on apoB mRNA editing, increasing concentrations of calcium ion, calcium ionophores (A23187 and ionomycin), and the Ca^{2+}-ATPase inhibitor thapsigargin were added to the culture media of McA7777 and Caco-2 cells and incubated at 37°C for 6 h. Following the treatment, apoB mRNA editing in the cells was analyzed. As shown in Fig. 1, the calcium ionophores A23187 and ionomycin increased the editing up to 2.3-fold at 10 μM in McA7777 cells. The calcium ionophores increased apoB mRNA editing up to 1.5-fold in Caco-2 cells in a dose dependent manner. Thapsigargin increased dose-dependently apoB mRNA editing up to 2- and 3-fold in Caco-2 and McA7777 cells, respectively, reaching a plateau at 50 and 200 nM respectively (Fig. 1). Thapsigargin depletes intracellular calcium stores and raises the cytoplasmic calcium concentration through inhibiting Ca^{2+}-ATPase (33). The strong stimulation by releasing endogenous calcium with thapsigargin together with the calcium ionophore effect suggest that calcium may play a role in apoB mRNA editing regulation.

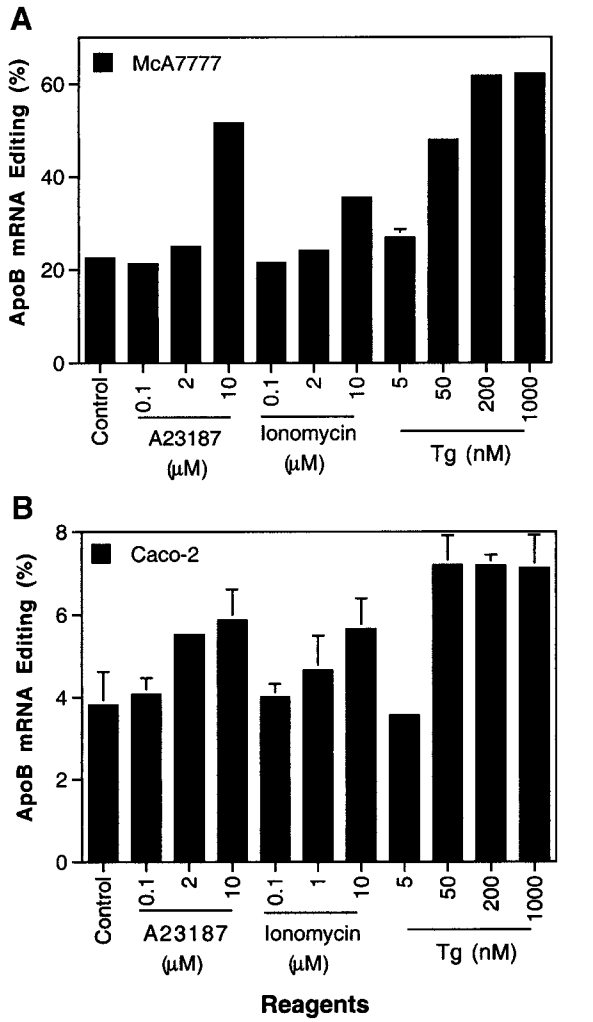

FIG. 1. Effect of calcium ionophore (A23187 and ionomycin) and Ca^{2+}-ATPase inhibitor thapsigargin on apoB mRNA editing in McA7777 (A) and Caco-2 (B) cells. Agents were added to the culture media to a final concentration indicated. The cells were further incubated at 37°C for 6 h. Total RNA was isolated and apoB mRNA editing was analyzed by RT-PCR followed by primer extension. The editing percentage stands for apoB-48/(apoB-48 + apoB-100). Tg stands for thapsigargin. The values are mean ± SD, and represent triplicate determinations of three different extract preparations.

The cell culture medium used for McA7777 and Caco-2 cells contained 1.8 mM calcium ion. Addition of calcium chloride to the medium showed that calcium had no effect on apoB mRNA editing with concentrations up to 20 mM in McA7777 or 10 mM in Caco-2. Thereafter, the editing increased dose-dependently up to 2.3-fold (Figs. 2A and 2B). The medium used for the calcium experiments contained 10 or 25% serum which contained calcium-binding proteins. To test if the lag phase effect of increasing extracellular calcium on apoB mRNA editing was due to partial sequestration of calcium ion by proteins from the serum, the calcium effect on apoB mRNA editing was also investigated with serum free medium. As shown in Figs. 2A and 2B, the calcium effect on apoB mRNA editing was similar when using medium with or without serum. However, with a high concentration of calcium (50 mM), a more potent effect was seen with serum free medium in Caco-2 cells. When increasing concentrations of calcium and 10 μM ionomycin were administered together, a synergistic stimulation effect on apoB mRNA

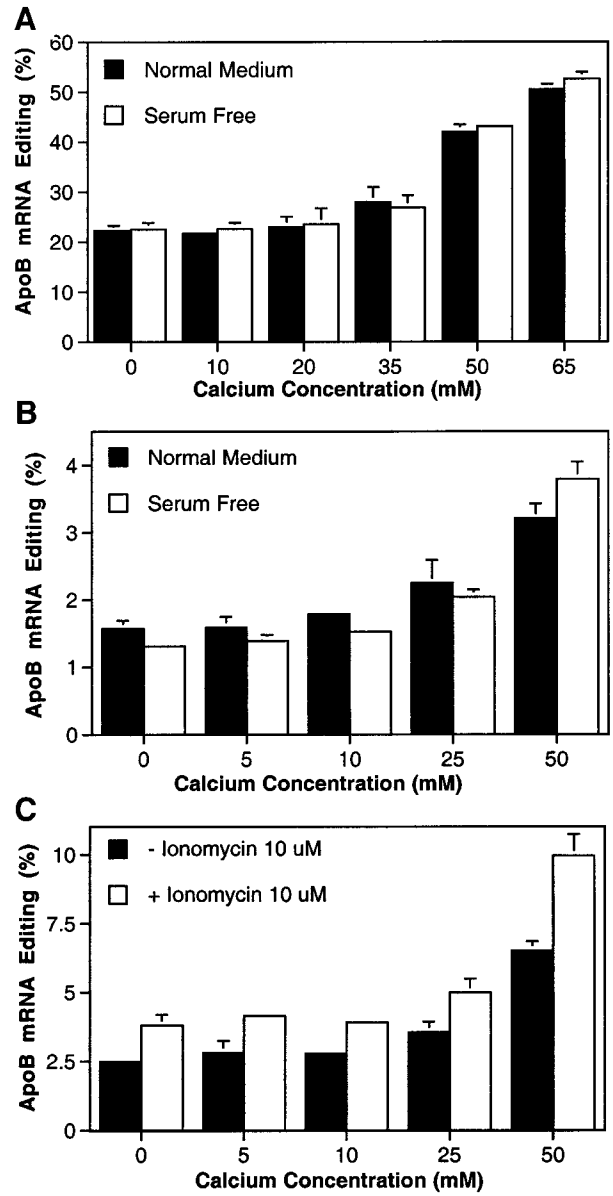

FIG. 2. Effect of increasing the extracellular calcium concentration on apoB mRNA editing in McA7777 and Caco-2 cells. Calcium chloride dissolved in water was added to the culture medium with or without serum to a final concentration indicated. The cells were further incubated at 37°C for 6 h and apoB mRNA editing was analyzed from the total cellular RNA extracted. (A) and (B) were calcium effects in McA7777 and Caco-2 cells, respectively. (C) Caco-2 cells treated with increasing calcium concentrations with or without 10 μM ionomycin. The values are mean ± SD, and represent triplicate determinations of three different extract preparations.

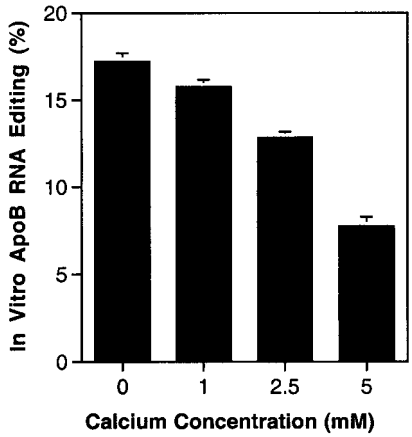

FIG. 3. Calcium effect on *in vitro* apoB RNA editing. Increasing concentrations of calcium chloride was included in the *in vitro* apoB RNA editing reaction as described under Materials and Methods. The reaction was incubated at 30°C for 3 h and the RNA was extracted. The apoB RNA editing was determined by primer extension after RT-PCR amplification. The values are mean ± SD, and represent triplicate determinations of three different extract preparations.

editing was observed (Fig. 2C), indicating that the stimulative effect of a high concentration calcium in medium was mediated through increasing the cytoplasmic calcium concentration.

Calcium stimulation effect on apoB mRNA editing is mediated through other cellular factors. An increase of cytoplasmic calcium by calcium ionophores and the Ca^{2+}-ATPase inhibitor thapsigargin stimulated apoB mRNA editing (Fig. 1). The editing can also be stimulated by increasing the calcium concentration in the cell culture medium but the stimulation occurred only at high concentrations (Fig. 2). To understand the possible mechanism, the calcium effect on *in vitro* apoB mRNA editing was performed with a rat liver protein extract. As shown in Fig. 3, calcium had an inhibitory effect instead of stimulatory effect on the editing, indicating that the calcium stimulatory effect on apoB mRNA editing in cells is not due to a direct calcium effect but mediated through cellular factors. A calcium effect for concentrations above 5 mM could not be determined because of RNA and perhaps protein degradation induced by high concentrations of calcium. To get further insight, the time dependence of 50 mM calcium effect on apoB mRNA editing in McA7777 cells was investigated. As shown in Fig. 4A, the calcium stimulating effect was observed after 1 h and increased gradually. Compared with the control, both apoB and APOBEC-1 mRNA levels remained unchanged until 8 h where the control APOBEC-1 and apoB mRNA levels increased (Figs. 4B and 4C). Similar results were also obtained for the time dependence of 50 mM calcium effect on apoB mRNA editing in Caco-2 cells and Hep G2 cells stably expressing human APOBEC-1 (data not shown). These data indicate that calcium increases apoB mRNA editing without any relationship to APOBEC-1 and apoB mRNA expression levels.

DISCUSSION

Calcium is involved in regulating apolipoprotein metabolism (34). Increasing calcium concentrations in the culture medium as well as treatment with the calcium ionophore A23187 or Ca^{2+}-ATPase inhibitor thapsigargin inhibits apoB-containing VLDL secretion in pri-

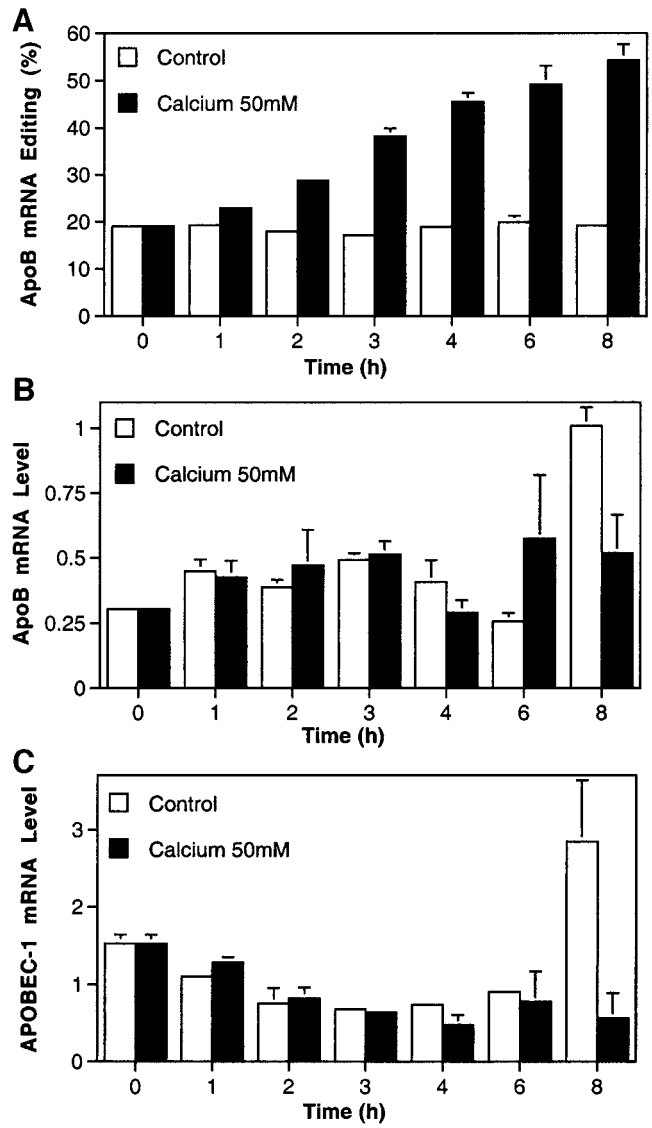

FIG. 4. Time course of extracellular calcium effect on apoB mRNA editing, apoB mRNA level, and APOBEC-1 mRNA level in McA7777 cells. McA7777 cells were incubated in media containing 50 mM calcium chloride at 37°C for the indicated duration. Total RNA was extracted and analyzed for apoB mRNA editing (A), apoB mRNA level (B), and APOBEC-1 mRNA level (C) as described under Materials and Methods. The values are mean ± SD, and represent triplicate determinations of three different extract preparations.

mary cultures of rat hepatocytes (26, 29). On the other hand, apoB synthesis and secretion in Caco-2 cells can be increased by A23187 but is unaltered by 50 mM calcium in the culture medium (24). In this paper, we demonstrate for the first time that calcium increases apoB mRNA editing in both Caco-2 and McA7777 cells. Treatments with the calcium ionophores (A23187 and ionomycin), the direct addition of calcium chloride in medium, and the Ca^{2+}-ATPase inhibitor thapsigargin all resulted in dose-dependent increases of apoB mRNA editing. A23187 and ionomycin form stable complexes with calcium, transport calcium across the cell membrane and thus increase the intracellular calcium concentration (35, 36). Thapsigargin releases endogenous calcium from intracellular stores and increases the cytoplasmic calcium concentration by inhibiting Ca^{2+}-ATPase (33). The stimulative effect of the ionophores (A23187 and ionomycin) and thapsigargin on apoB mRNA editing strongly suggest that calcium increases apoB mRNA editing and has a regulatory role.

Calcium is required for life, yet prolonged high intracellular calcium levels lead to cell death. Cells tightly regulate intracellular calcium level through numerous binding and specialized extrusion proteins because calcium cannot be metabolized like other molecules (37). This may be the reason why extracellular calcium concentration up to 10 and 20 mM has no effect on apoB mRNA editing in Caco-2 and McA7777 cells respectively (Fig. 2). With high concentrations of calcium in the media, apoB mRNA editing is increased most likely by diffusion mediated by increased intracellular calcium. In support of this mechanism is the synergistic stimulatory effect of ionomycin and high concentrations of extracellular calcium (50 mM) on apoB mRNA editing. Since ionomycin functions by transporting calcium into cells, the synergistic stimulatory effect occurred only at a high extracellular calcium (50 mM) indicates that the effect is not simply mediated by providing additional calcium for ionomycin to transport but 50 mM extracellular calcium has itself calcium diffusion to increase intracellular calcium to generate a synergistic calcium increase with ionomycin.

The direct addition of calcium chloride to an *in vitro* apoB mRNA editing reaction using a rat liver protein extract resulted in inhibition instead of the stimulation of the editing as was observed in intact cells. This observation could reflect, at least in part, increased degradation of the apoB mRNA and/or APOBEC-1 protein. This finding suggests that calcium has no direct effect on the editing enzyme complex and that the stimulatory effect on apoB mRNA editing is mediated through the other cellular factors which in turn regulate the apoB mRNA editing enzyme activity. The finding that calcium increases apoB mRNA editing without APOBEC-1 and apoB mRNA expression level changes during the first six hours (Fig. 4) suggests that the apoB mRNA editing enzyme may be posttranslationally activated. Alternatively the effect could also be mediated by an effect upon the other protein components of the editing complex which changes the component expression levels.

The calcium antagonists verapamil and diltiazem also inhibit VLDL-associated apoB and triacylglycerol secretion in rat hepatocyte primary cultures (25, 27). However, in our studies, no effect on apoB mRNA editing was found for diltiazem and low concentrations of verapamil. With a high concentration (200 μM) of verapamil, apoB mRNA editing increased by twofold in both Caco-2 and McA7777 cells (data not shown). Since verapamil and diltiazem bind to calcium channels and may exert their effects through mediator(s) other than cellular calcium concentration, it is not known if the stimulation resulted from altered calcium levels (38).

LDL can transiently increase cytoplasmic calcium concentration (23). Hormones including adrenaline (39), noradrenaline (40), phenylephrone (29), and vasopressin (29) can cause indirect calcium mobilization and are reported to suppress VLDL metabolism. However, none of these agents had an effect on apoB mRNA editing (data not shown). This may reflect the transient nature of the calcium increase by these agents as well as the limitation of using apoB mRNA editing as the detection assay. Since the proportion of edited apoB mRNA in a pre-existing apoB mRNA pool is evaluated, the editing enzyme activity alteration may be rapid in onset but the down-stream editing change in apoB mRNA may require a longer period of time or a greater magnitude of effect in order to be detectable.

In summary, we demonstrate that (a) high concentrations of extracellular calcium stimulates apoB mRNA editing without altering apoB or APOBEC-1 mRNA expression; (b) increasing intracellular calcium by calcium ionophores (A23187 and ionomycin) and the Ca^{2+}-ATPase inhibitor thapsigargin stimulate apoB mRNA editing; and (c) calcium has no direct effect on apoB mRNA editing as shown in an *in vitro* editing reaction, suggesting that the mediation is through other factors present in an intact cells. Taken together, these data indicate that calcium plays a role in regulating apoB mRNA editing.

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