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Effects of Expression of a Mouse Brain L-Type Calcium Channel α1 Subunit on Secretion from Bovine Adrenal Chromaffin Cells

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SUMMARY

Regulated exocytosis from bovine chromaffin cells is stimulated by the influx of Ca^{2+} through plasma membrane ion channels that are opened by nicotinic stimulation and/or depolarization. Recently, we developed a novel method that enabled us to investigate the function of a cloned Ca^{2+} channel type C α1 subunit in forming channels that stimulate exocytosis. In the present study, we demonstrate by immunocytochemistry that bovine chromaffin cells normally express an epitope specific for the type C α1 subunit. We investigated the effects of expression of additional class C α1 subunits (mouse brain clone) on various aspects of secretory function in bovine chromaffin cells by measuring secretion of cotransfected human growth hormone (GH, a reporter for the regulated secretory pathway in the transfected cells). New channels were activated in response to depolarization by both elevated K^+ and nicotinic cholinergic agonist. The new channels had their greatest effects when secretion was stimulated suboptimally. Secretion was enhanced only after the first 30 sec of stimulation, and the enhancement extended beyond 5 min of continuous stimulation. In contrast to the endogenous L-type Ca^{2+} channels, the latency was not decreased by the dihydropyridine L-type Ca^{2+} channel agonist, Bay K 8644. The findings suggest that (i) the Ca^{2+}-sensitive mechanism for triggering or maintaining exocytosis is capable of being saturated by high levels of Ca^{2+}, (ii) secretion caused by nicotinic agonist stimulation can be significantly enhanced by activation of voltage-sensitive Ca^{2+} channels, and (iii) the effects on secretion of the L-type Ca^{2+} channels formed on expression of the mouse brain class C α1 subunit are distinct from those of endogenous L-type Ca^{2+} channels.

One of the primary functions of voltage-sensitive Ca^{2+} channels in nerve terminals and other excitable secretory cells is the regulation of Ca^{2+} influx that triggers exocytosis. The modulation of these channels is central to the physiological processes that regulate the secretory function of the cells. Electrophysiological, pharmacological, and biochemical experiments have been used to identify multiple types of Ca^{2+} channels with distinct voltage-gating characteristics, pharmacology, and abilities to couple Ca^{2+} influx to exocytosis (1–7). The purpose of the present study was to investigate the role of a specific L-type Ca^{2+} channel in regulating secretion.

Protein purification and molecular cloning have revealed substantial complexity and diversity of Ca^{2+} channels (1, 2, 8, 9). The subunit structure of the L-type, dihydropyridine-sensitive channel in skeletal muscle is probably the best understood (1, 2, 10). It is a pentamer composed of α1, α2, δ, β, and γ subunits. The α1 subunit (175–210 kDa) comprises the pore and contains four homologous regions, each with six transmembrane domains. It also contains dihydropyridine- and verapamil-binding sites. Expression of the α1 subunit alone results in channel activity but is not sufficient to reconstitute normal Ca^{2+} channel function with rapid kinetics. Expression of the α1 subunit together with the β subunit permits expression of Ca^{2+} channels that are more physiological (11–13).

Bovine chromaffin cells are excitable, neuroendocrine cells. Ca^{2+} influx through the nicotinic receptor/channel complex raises intracellular Ca^{2+} and triggers exocytosis of chromaffin granules. These secretory granules contain catecholamine, ATP, opiate peptides, and a variety of proteins. Ca^{2+} channels in bovine chromaffin cells have been intensively investigated with the use of both biochemical (14–17) and electrophysiological (18–22) techniques. There is strong evidence for the existence of L-, Q-, N-, and P-type channels, all of which can trigger exocytosis, with the L-type channel being most efficient (6, 23). With brief depolarizations to ~0

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ABBREVIATIONS: GH, human growth hormone; PSS, physiological salt solution; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

mV in the absence of pharmacological agents, a "standard Ca^{2+} current" (21) is observed that is mainly accounted for by 14-pS conductance channels consisting of N- and P-type channels. L-type Ca^{2+} channels do not open readily and do not contribute significantly to the divalent ion currents induced by short depolarization. L-type Ca^{2+} channels are activated by prolonged or repetitive depolarizations. The activation of these channels accounts for the facilitation current that was first observed by Fenwick *et al.* (18) and later characterized by Artalejo *et al.* (20). The current progressively increases during 1-Hz stimulation (20-msec depolarizations to 0 mV). Prepulses to +120 mV for 200 msec completely activate the facilitation current (21). There is evidence that protein phosphorylation is directly or indirectly involved in causing the facilitation current (22, 24). During continuous depolarization to −10 mV (a potential achieved by elevation of extracellular K^+ to 56–100 mM), opening of a significant number of L-type Ca^{2+} channels is not observed until after 20 sec. Channel opening reaches a maximum by 40 sec and declines by 1 min (20). Preincubation with the dihydropyridine Ca^{2+} channel agonist Bay K 8644 induces immediate and long-lived opening of the 27-pS channel on subsequent depolarizations without the requirement of large or repetitive predepolarizations (20).

The role of specific Ca^{2+} channels in Ca^{2+}-dependent exocytosis has been investigated primarily with pharmacological agents specific for different classes of channels and with carefully controlled depolarizations paradigms in electrophysiological experiments. Recently, we developed a novel method that enabled us to investigate the function of a cloned Ca^{2+} channel $\alpha 1$ subunit in forming channels that stimulate exocytosis (9). Bovine chromaffin cells were transfected with a vector (pCMV.MC1) that encodes a class C isoform of the $\alpha 1$ subunit of L-type Ca^{2+} channel from mouse brain. We found that expression of the isoform conferred properties to bovine chromaffin cells consistent with the formation of new Ca^{2+} channels. Secretion of cotransfected human GH (a reporter for the regulated secretory pathway in the transfected cells) was enhanced in response to depolarization (9). The enhancement was sensitive to the dihydropyridine agonist Bay K 8644 and the phenylalkylamine Ca^{2+} channel antagonist D-600, consistent with the new channels being of the L-type. Because in bovine chromaffin cells secretion is correlated with Ca^{2+} influx, it is likely that increased secretion results from an increased number of channels composed of the transiently expressed mouse brain $\alpha 1$ subunit and endogenous subunits that are also necessary to create a physiologically functional channel.

The following experiments are the first in which the functional consequences of additional Ca^{2+} channels in secretory cells were examined in detail. We found by using immunocytochemistry that chromaffin cells express an epitope specific for class C $\alpha 1$ subunit of L-type Ca^{2+} channels. Transiently expressed mouse class C $\alpha 1$ subunits had their greatest effects when secretion was stimulated suboptimally and could enhance secretion induced by nicotinic stimulation as well as by elevated K^+. The new channels enhanced secretion after only 30 sec of depolarization. The Ca^{2+} channels created by expression of the exogenous class C $\alpha 1$ subunits had properties that distinguish them from the endogenous L-type channels.

Materials and Methods

Chromaffin cell culture and transfection. Methods for preparation of monolayer cultures of purified, primary dissociated chromaffin cells from bovine adrenal medulla; for transfection with calcium phosphate; and for measurement of secretion are identical to those of a previous study (25). Cotransfections with pXGH5 encoding GH (26) and either pCMV.MC1 (encoding the mouse brain class C $\alpha 1$ subunit) (9) or pCMV.neo (the parent plasmid for pCMV.MC1) were performed within 24 hr of isolation of the cells. Secretion experiments were performed 4–6 days after transfection. In previous experiments, we determined that ≥75% of the GH-expressing cells coexpress a cotransfected plasmid (27). Because transfection of nonpurified and purified cells gave similar results, some experiments were performed on nonpurified cells.

Secretion experiments. Chromaffin cells were washed before each experiment with PSS (145 mM NaCl, 5.6 mM KCl, 2.2 mM $CaCl_2$, 0.5 mM $MgCl_2$, 15 mM HEPES, pH 7.4., 5.6 mM glucose, and 0.5 mM ascorbate) (28). GH secretion from the small fraction of transfected cells and catecholamine secretion from the large fraction of untransfected cells were measured. Depolarization-induced secretion was investigated in elevated K^+-containing solution (PSS containing either 56 mM KCl and 94.6 mM NaCl or 24 mM KCl and 126.6 mM NaCl). Free Ca^{2+} in nitrilotriacetic acid-buffered solutions was calculated according to Chang *et al.* (29) using constants from Martell and Smith (30) and was confirmed with a Ca^{2+}-sensitive electrode. GH released into the medium and retained by the cells was measured with either a very sensitive radiometric, immunological assay or an even more sensitive chemiluminescent assay, both from the Nichols Institute (San Juan Capistrano, CA). Endogenous catecholamine released into the medium and retained by the cells was measured with a fluorescence assay (31).

Calcium uptake studies. Purified chromaffin cells (180,000/6.8-mm well) were preincubated for 30 sec in buffer containing 4 mM K^+ and 2.2 mM Ca^{2+}, with or without 1 μM Bay K 8644. Cells were then incubated with buffer containing either 5.6 mM or 24 mM K^+ and 2.2 mM Ca^{2+} [2 μCi/ml $^{45}CaCl_2$, 13.7 mCi/mg Ca stock (ICN, Irvine, CA)] with or without 1 μM Bay K 8644 for 30 sec. These solutions were aspirated after 30 sec and replaced with ice-cold nondepolarizing PSS containing 1 mM La^{3+}, with each well subsequently washed three times with this buffer. A solution (100 μl) containing 1% Triton X-100 and 1 mM EGTA was then added to each well. The radioactivity taken from each well was counted in 4 ml Universol (ICN, Irvine, CA) scintillation counting solution. Data were expressed in p[M of Ca^{2+} taken up by 180,000 cells.

Immunocytochemistry. Immunocytochemistry on fixed and methanol-permeabilized chromaffin cells was performed as previously described (27) to detect type C $\alpha 1$ subunits of L-type Ca^{2+} channels in bovine chromaffin cells. Antibody to the type C $\alpha 1$ subunit (CNC1) was produced in rabbits against the highly variable site in the intracellular loop between domains II and III of rat $\alpha 1$ subunits of Ca^{2+} channels (32). The sequence is completely conserved in all isoforms of neuronal class C $\alpha 1$ subunits that are generated by alternative splicing (33). Goat anti-dopamine-β-hydroxylase was used to identify chromaffin cells. The primary antibodies were detected by donkey, FITC-anti-goat, and donkey, lissamine rhodamine anti-rabbit antibodies. Specific binding of the CNC1 antibody was determined by comparison of the images with those obtained with antibody blocked by an overnight incubation with the specific peptide (25 μM).

Plasmids. GH was expressed using XGH5, in which GH expression is driven by a mouse metallothionein-I promoter (26). Adequate expression was obtained without incubation with heavy metals. The GH expression was variable, ranging between 0.3 and 2 ng/35-mm well and between 0.5 and 3 ng/60-mm dish. The class C isoform of the mouse brain $\alpha 1$ subunit was expressed using pCMV.MC1 as described previously (9). The control for pCMV.MC1 was pCMV.Neo, the parent plasmid.

Data analysis. Data are expressed as the percentage of total secreted GH or catecholamine in cells (mean ± standard error). Significance was tested with the use of Student's *t* test.

Results

Immunocytochemistry suggests that class C α1 subunit isoform of L-type Ca^{2+} is expressed in bovine chromaffin cells. CNC1 antibody is directed against an intracellular domain of the α1 subunit of the class C rat brain, L-type Ca^{2+} channel and is highly specific (32, 34). The antibody specifically stained bovine chromaffin (DBH-positive cells) (Fig. 1, A and B). Staining was blocked by prior incubation of the affinity-purified antibody with the CNC1 peptide (Fig. 1, C and D) against which the antibody was generated but not by prior incubation with CND peptide (data not shown). Staining was apparent throughout the cell. In some views (not apparent in the photographs), fine speckling appeared to be associated with the cell surface.

Effects of expressing the mouse brain class C α1 subunit on the time course of secretion in response to K^+-induced depolarization. Previous research has shown that chromaffin cells transfected with pCMV.MC1 encoding a mouse brain class C α1 subunit release approximately twice as much GH into the medium as do control cells over a continuous 15-min incubation with 20 mM K^+ (9). To further examine the coupling of the exogenous α1 subunit-derived channel to secretion, we investigated the effects of transfection with the α1 subunit on the time course of GH secretion induced by raising the K^+ concentration from 5.6 mM to either 24 or 56 mM. The membrane potential shifts from a resting potential of ~−55 mV to −30 mV in 24 mM K^+ and to −15 mV in 56 mM K^+ (35). The earliest time point at which we could precisely measure stimulated secretion was 30 sec. Secretions occurring between 30 sec and 5 min and between 5 min and 15 min were also measured.

After 30 sec of depolarization, pCMV.MC1-transfected cells secreted GH to a greater extent than control cells in response to both 24 mM K^+ (Fig. 2A) and 56 mM K^+ (Fig. 2B). The relative enhancement and sometimes the absolute increase of secretion were larger with the suboptimal K^+ concentration. For example, cells expressing the mouse brain class C α1 subunit secreted 3-fold more GH (an additional 6% of the total cellular GH) than did pCMV.Neo-transfected cells in response to 24 mM K^+ from 30 sec to 5 min. In contrast, cells expressing the mouse brain class C α1 subunit secreted only 25% more GH secretion (an additional 3% of the total cellular GH) than did pCMV.Neo-transfected cells in response to 56 mM K^+ over the same interval. Catecholamine release is predominantly a measure of secretion from nontransfected cells since nontransfected cells represent the vast majority of the cell on the dish. As expected, catecholamine secretion was similar in pCMV.MC1 and pCMV.Neo cultures (data not shown).

There was no effect of the mouse brain class C α1 subunit-derived channels before 30 sec with either 24 mM K^+ or 56 mM K^+. It is possible that at 56 mM K^+, Ca^{2+} influx maximally stimulates secretion in the absence of exogenous Ca^{2+} channels so that additional channels have no effect. However, this could not be the case at 24 mM K^+, where secretion from 0 to 30 sec is much less than that during the same interval at 56 mM K^+.

Nicotinic stimulation of GH release is increased by expressing the mouse brain class C α1 subunit. The normal physiological stimulus for bovine chromaffin cells is acetylcholine acting through nicotinic receptors on the chromaffin cell plasma membrane. This nicotinic channel is of the neuronal type that is permeable to Ca^{2+} as well as Na^+ (36). Because nicotinic stimulation depolarizes the cell, Ca^{2+} influx that couples to secretion could also occur through voltage-sensitive Ca^{2+} channels, including L-type channels. It is unclear in pharmacological experiments the degree to which voltage-sensitive Ca^{2+} channels contribute to the secretion in response to a nicotinic agonist. If influx through voltage-sensitive Ca^{2+} channels can affect the secretory response

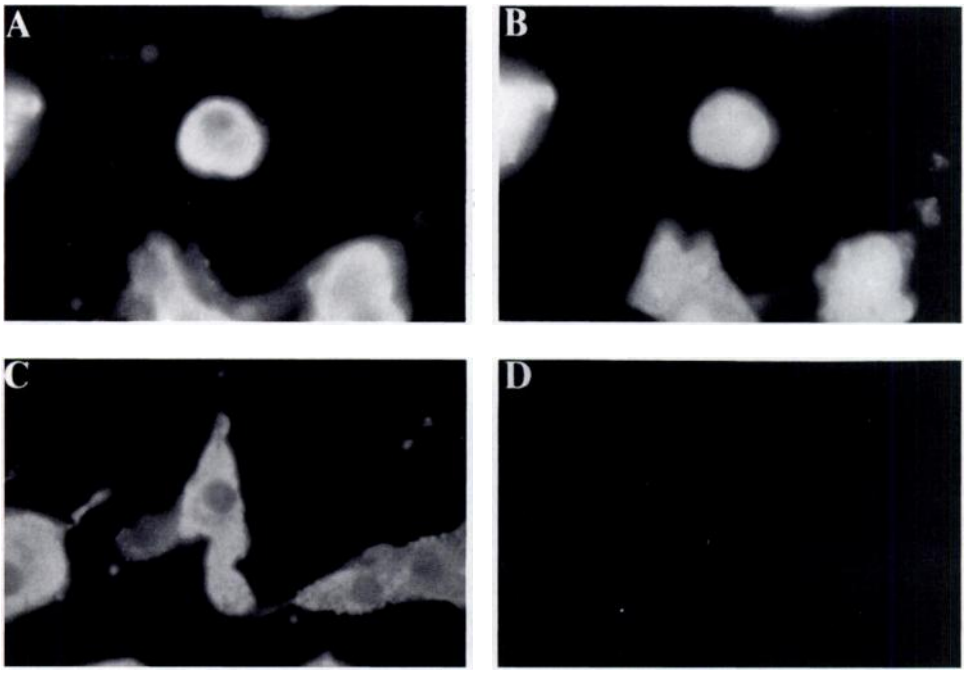

Fig. 1. The class C α1 subunit isoform of L-type Ca^{2+} is expressed in bovine chromaffin cells. Fixed and methanol permeabilized chromaffin cells were incubated with rabbit anti-C α1 (followed by lissamine rhodamine-donkey anti-rabbit antibody) and goat anti-dopamine-β-hydroxylase (followed by FITC-donkey anti-goat antibody). A and C, Dopamine-β-hydroxylase. B, C α1 subunit. D, Anti-C α1 antibody had been incubated with the peptide against which the antibody had been generated before incubation with the cell. The specificity of the immunocytochemistry is demonstrated by comparison of B and D.

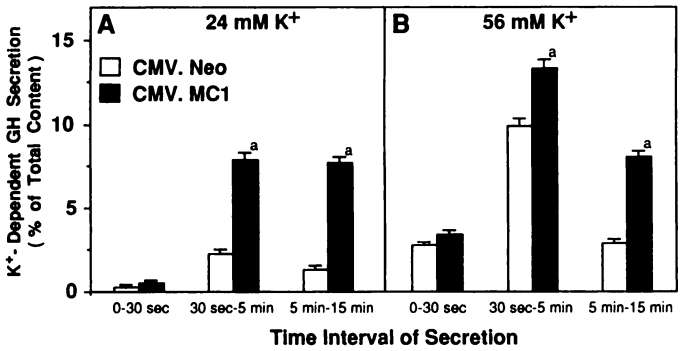

Fig. 2. Expression of the mouse brain class C isoform of the a1 subunit in chromaffin cells enhances GH release in response to elevated K^+ depolarization. Bovine adrenal chromaffin cells were cotransfected with the combination of pXGH5 and pCMV.Neo [control (*open bars*)] or pXGH5 and pCMV.MC1 (*black bars*). At 4–6 days after transfection, secretion was determined for cells incubated in either 24 mM K^+ (A) or 56 mM K^+ (B) for the indicated serial time intervals. The data are pooled results from five experiments with a total of 18 dishes/group for the 24 mM K^+ data and 14 dishes/group for the 56 mM K^+ experiments. After 30 sec, GH secretion from cells transfected with pCMV.MC1 is significantly greater than the pCMV.Neo control. *a*, $p < 0.001$ versus pCMV.Neo.

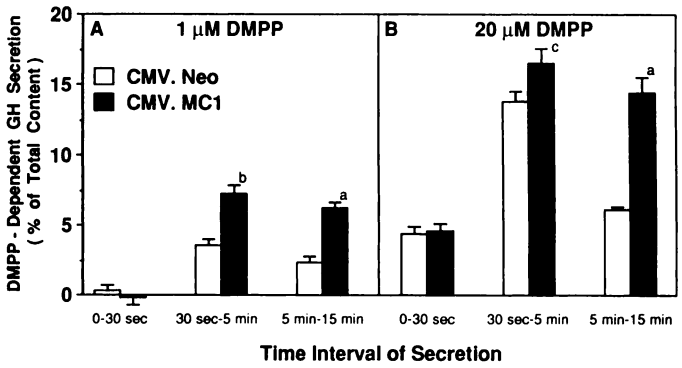

Fig. 3. Exogenous mouse brain class C α1 subunit expression increases the GH secretory response to nicotinic stimulation. Bovine adrenal chromaffin cells were cotransfected with the combination of pXGH5 and pCMV.Neo or pXGH5 and pCMV.MC1. At 4–6 days after transfection, secretion was determined for cells incubated in either 1 μM DMPP (A) or 20 μM DMPP (B) for the indicated serial time intervals. There were four dishes/group for the 1 μM DMPP data and eight dishes/group for the 20 μM DMPP experiments. *a*, $p < 0.001$; *b*, $p < 0.01$; *c*, $p < 0.05$ versus pCMV.Neo.

during nicotinic agonist-induced stimulation and depolarization, then additional channels created by expressing the mouse brain class C α1 subunit should enhance the secretory response.

During the intervals between 30 sec and 5 min and 5 to 15 min, cells expressing the mouse brain class C α1 subunit secreted more GH in response to both 1 and 20 μM DMPP than did cells transfected with pCMV.Neo (Fig. 3, A and B). These data show that the exogenous Ca^{2+} channels can be opened with nicotinic stimulation and that the additional Ca^{2+} influx can enhance secretion. The relative enhancement of GH secretion in the middle time interval (30 sec to 5 min) caused by expressing the α1 subunit was greater for 1 μM DMPP than it was for 20 μM DMPP. As with depolarization with elevated K^+, no effect of the exogenous α1 subunit was evident before 30 sec with either 1 μM or 20 μM DMPP.

Expression of the mouse brain class C α1 subunit enhances secretion to a greater extent when the extracellular Ca^{2+} is reduced. In the following experiment, secretion was limited by lowering the extracellular Ca^{2+}, thereby reducing Ca^{2+} influx through individual channels (Fig. 4B), rather than by lowering the stimulus and thereby reducing the number of open channels. Cells were depolarized with 56 mM K^+ in the presence of 0.2 mM or 2.2 mM Ca^{2+}. Although the exogenous α1 subunits were able to enhance secretion at both high and low Ca^{2+} concentrations, the relative stimulatory effects were greater at the lower Ca^{2+} concentration. The mouse brain α1 subunit did not enhance GH secretion as strongly when the extracellular Ca^{2+} concentration was limiting secretion as it did when the secretory response was limited by weak depolarization. For example, from 30 sec to 5 min, the mouse brain α1 subunit caused a 52% increase in GH secretion stimulated by 56 mM K^+ and 0.2 mM Ca^{2+} (Fig. 4B). In contrast, cells expressing the α1 subunit secreted three times as much GH when pCMV.MC1-transfected cells were exposed to 24 mM K^+ in 2.2 mM Ca^{2+} during the same interval (Fig. 2B). There was little effect of the exogenous channels on GH secretion in the first 30 sec.

Bay K 8644 does not reduce the latency of the enhancement of secretion caused by new Ca^{2+} channels. One explanation for the absence of effects on secretion before 30 sec is that the new channels do not open before 30 sec. Electrophysiological experiments indicate that endogenous L-type Ca^{2+} channels in chromaffin cells open on depolarization to −10 mV after a latency of 20 sec (see introductory paragraphs). If the new and endogenous channels function similarly, then preincubation with the L-type Ca^{2+} channel agonist Bay K 8644 should eliminate the latency of channel opening in response to depolarization (20). We examined the effects of 1 μM Bay K 8644 on the early rates of catecholamine secretion and Ca^{2+} uptake in nontransfected chromaffin cell cultures. We then investigated the effects of this pretreatment on the latency of the effects of the exogenous channels on secretion.

Nontransfected chromaffin cells were preincubated with or without 1 μM Bay K 8644 in 4 mM K^+-containing solution for 30 sec before incubation of the cells with 24 mM K^+. The

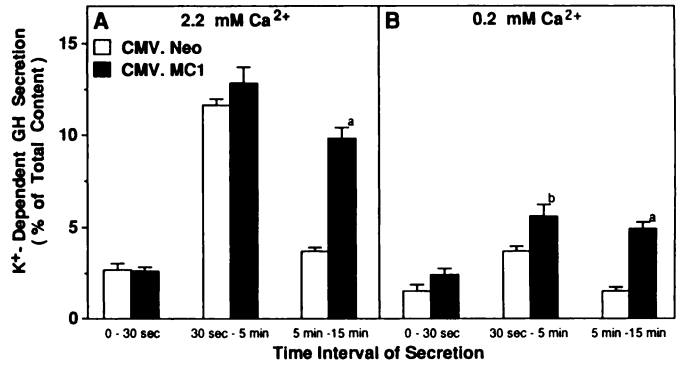

Fig. 4. Expression of the mouse brain class C a1 subunit reduces the effects of lowered extracellular Ca^{2+} levels on exocytosis from chromaffin cells. Bovine adrenal chromaffin cells were cotransfected with the combination of pXGH5 and pCMV.Neo or pXGH5 and pCMV.MC1. At 4–6 days after transfection, secretion was determined for cells incubated in 56 mM K^+ buffer with either 2.2 mM Ca^{2+} (A) or 0.2 mM Ca^{2+} (B) for the indicated serial time intervals. Data are the pooled results from three experiments for a total of 12 dishes/group. *a*, $p < 0.001$; *b*, $p < 0.01$ versus pCMV.Neo.

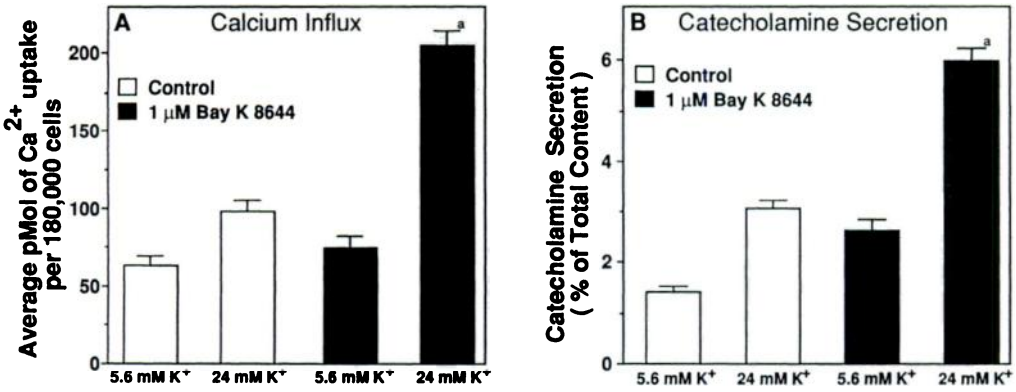

Fig. 5. Bay K 8644 enhances both depolarization-dependent calcium influx (A) and catecholamine release (B) from bovine chromaffin cells before 30 sec. Untransfected bovine adrenal chromaffin cells were preincubated for 30 sec in buffer containing 4 mM K^+ and 2.2 mM Ca^{2+} with or without 1 μM Bay K 8644. Cells were then stimulated by incubation with buffer containing either 5.6 mM or 24 mM K^+ and 2.2 mM Ca^{2+} with or without 1 μM Bay K 8644 for 30 sec. Ca^{2+} influx and catecholamine secretion were measured as described in Materials and Methods. Ca^{2+} influx data are combined from four experiments with a total of 18 wells/group. Catecholamine secretion data are combined from four experiments with a total of 32 dishes/group. *a*, $p < 0.001$ versus treatment with 24 mM K^+ alone.

combination of 1 μM Bay K 8644 and 24 mM K^+ tripled the amounts of $^{45}Ca^{2+}$ uptake (Fig. 5A) and catecholamine secretion (Fig. 5B) induced by incubation for 30 sec with 24 mM K^+. Enhancement by Bay K 8644 of depolarization-induced Ca^{2+} uptake was significant as early as 10 sec ($p < 0.001$), was completely reversed by 3 μM nifedipine, and was not altered by the combination of 2 μM ω-conotoxin GVIA and 200 nM ω-agatoxin IVA (data not shown).

These data confirm electrophysiological experiments (6) that show that preincubation with Bay K 8644 causes endogenous L-type Ca^{2+} channels to open more rapidly on depolarization to increase early Ca^{2+} influx and catecholamine secretion. To determine whether the new Ca^{2+} channels behave identically to the endogenous L-type facilitation channels, the effects of pretreatment with Bay K 8644 on GH secretion in pCMV.MC1-transfected cells were investigated. If the exogenous channels act as endogenous facilitation channels, then they should open immediately on depolarization after preincubation with Bay K 8644, thereby causing a greater Bay K 8644-induced enhancement of GH secretion in the first 30 sec. Transfected cells were preincubated with and without Bay K 8644 and then stimulated to secrete in 24 mM K^+ in the continuing presence or absence of Bay K 8644. The free Ca^{2+} concentration was 100 μM Ca^{2+} (buffered with nitrilotriacetic acid) rather than 2.2 mM to limit Ca^{2+} influx and better reveal effects of the exogenous channels. Bay K 8644 without depolarization did not cause significant secretion (data not shown). The drug significantly enhanced depolarization-induced GH (and catecholamine) secretion at all time points. Expression of the mouse brain α1 subunit increased the stimulatory effect of Bay K 8644 on GH secretion between 30 sec and 5 min by 60% and between 5 min and 15 min by 130%. However, expression of the exogenous α1 subunit did not significantly enhance Bay K 8644-induced secretion between 0 and 30 sec. In contrast, between 0 and 30 sec Bay K 8644 increased both GH secretion in transfected cells without the exogenous α1 subunit (Fig. 6A, with pCMV.Neo) and catecholamine secretion (mainly) from nontransfected cells in the culture (Fig. 6B, with both pCMV.Neo and pCMV.MC1). The expression of the exogenous α1 subunits did not enhance secretion between 0 and 30 sec in the presence of Bay K8644 in other experiments when cells were stimulated with 24 mM or 56 mM K^+ in the presence of 2.2 mM Ca^{2+} (data not shown).

Discussion

We previously demonstrated that a cloned splice variant class C α1 subunit had properties consistent with it forming new Ca^{2+} channels in bovine chromaffin cells. In the present study, we demonstrated that bovine chromaffin cells endogenously express an epitope specific for class C α1 subunit of L-type Ca^{2+} channels, and we investigated the consequences on the secretory response of additional channels formed upon expression of mouse brain class C α1 subunits. The effects of the exogenous channels were readily apparent despite a background of endogenous voltage-sensitive Ca^{2+} channels and could be studied without the need to inhibit by pharmacological blockers endogenous N and P type channels. Three findings have important implications for the understanding of the function of Ca^{2+} channels in regulating exocytosis: (i) the relative effects of the exogenous α1 subunit-derived Ca^{2+} channels were greater under suboptimal stimulating conditions compared with a maximal stimulus, (ii) the exogenous channels enhanced nicotinic agonist- as well as elevated K^+-induced secretion, and (iii) the effects of the extra Ca^{2+} channels on GH secretion were seen only after 30 sec of stimulation.

The exogenous channels have a more pronounced effect when chromaffin cells are suboptimally stimulated. The effects of additional Ca^{2+} channels on the secretory response of chromaffin cells to elevated K^+ or a nicotinic agonist are probably caused by increased Ca^{2+} influx at a given level of depolarization. In addition, there may be a somewhat greater stimulus-induced depolarization because of the larger resulting Ca^{2+} conductance. The relative increase and often the absolute increase in GH secretion were greater for the pCMV.MC1-transfected cells exposed to a mild rather than a strong stimulus. This was particularly evident during the interval from 30 sec to 5 min and occurred with either elevated K^+ (Fig. 2) or nicotinic agonist (Fig. 3). Expression of the exogenous α1 subunit caused a ≥2-fold increase in GH secretion from 30 sec to 5 min in submaximally stimulated cells, which is in contrast to a 20–30%

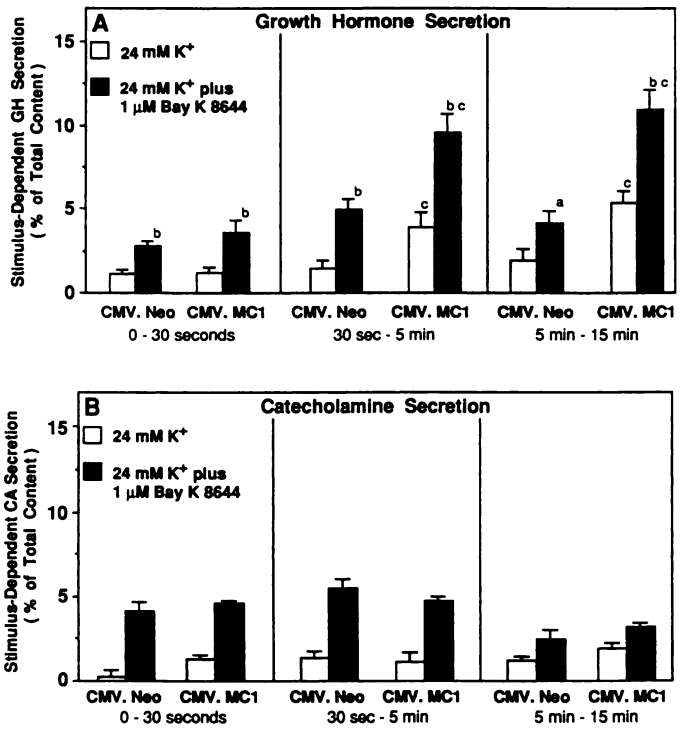

Fig. 6. Chromaffin cells expressing the mouse brain class C $\alpha 1$ subunit do not show increased early GH secretion when exposed to elevated K^+ and Bay K 8644. Bovine adrenal chromaffin cells were cotransfected with the combination of pXGH5 and pCMV.Neo or pXGH5 and pCMV.MC1. Secretion experiments were carried out 4–6 days after transfection. All rinse and incubation solutions contained 5 mM nitrilotriacetic acid (pH 7.0) to buffer the free Ca^{2+} concentration to 100 μM. Cells were preincubated for 30 sec in buffer containing 4 mM K^+ and 100 μM free Ca^{2+} with or without 1 μM Bay K 8644. Cells were then incubated with solution containing either 5.6 mM or 24 mM K^+ with or without 1 μM Bay K 8644 for the indicated serial time intervals. The releases of GH (A) and catecholamine (B) are depicted. Data are the pooled results from two experiments with a total of 10 dishes/group for the catecholamine data and 10 dishes/group for the GH data. Catecholamine release from identically treated pCMV.Neo- and pCMV.MC1-transfected cells was similar in all time intervals. Secretion in the absence of stimulation was 2.5–4% for all groups and times. Catecholamine release from the groups treated with combined 24 mM K^+ and 1 μM Bay K 8644 was significantly higher than that from the groups treated with 24 mM K^+ alone in all time intervals ($p < 0.05$). *a*, $p < 0.05$; *b*, $p < 0.01$ versus the same group treated with 24 mM K^+ alone; *c*, $p < 0.01$ versus the similarly treated pCMV.Neo group.

increase in secretion in maximally stimulated cells. The results may reflect the ability of the Ca^{2+}-sensitive mechanism involved in triggering or maintaining exocytosis to be saturated by high levels of Ca^{2+} influx. A similar conclusion was inferred from a previously report (31).

Between 5 and 15 min, the rate of secretion is normally greatly reduced because of lower rates of Ca^{2+} influx (31, 37). However, transfection with pCMV.MC1 resulted in robust enhancement of secretion during this time period (Figs. 2–4). Removal of the depolarizing stimulus and extracellular Ca^{2+} after 5 min halted subsequent GH secretion from pCMV.MC1-transfected cells (data not shown). Thus, it is likely that the effects of the exogenous $\alpha 1$ subunit result from continuing Ca^{2+} influx.

Nicotinic stimulation can enhance secretion by activating voltage-sensitive Ca^{2+} channels. Chromaffin cells have a neuronal-type nicotinic receptor/channel that has a significant Ca^{2+} permeability (36), which is sufficient to trigger exocytosis (38). It is uncertain whether depolarization induced by nicotinic stimulation causes activation of voltage-sensitive Ca^{2+} channels that contributes significantly to the secretory stimulus. Pharmacological approaches give ambiguous results because of difficulty in determining whether agents used to block voltage-sensitive Ca^{2+} channels are without effect on nicotinic receptor/channel function. The ability of expression of the mouse brain class C $\alpha 1$ subunit to enhance nicotinic agonist-induced secretion demonstrates that voltage-sensitive Ca^{2+} channels can contribute to the stimulation of exocytosis initiated by nicotinic receptor/channel activation. Results of the present study, therefore, suggest that in the intact gland during intense sympathetic discharge, secretion stimulated by acetylcholine released from preganglionic nerves is likely to have contributions from Ca^{2+} influx both through the nicotinic receptor/channel and through endogenous, voltage-sensitive Ca^{2+} channels.

The absence of effects on early secretion of expression of the mouse brain class C $\alpha 1$ subunit indicates the formation of a Ca^{2+} channel with novel characteristics. Additional channels that result from expression of the mouse brain class C $\alpha 1$ subunit did not significantly enhance secretion between 0 and 30 sec of stimulation. The delay may have been caused by a delay in the opening of the new channels. Endogenous L-type Ca^{2+} channels in bovine chromaffin cells also have similar delays in opening on depolarization to -10 mV (approximately the potential attained with 56 mM K^+) (20). The latency in the opening of endogenous L-type channel can be reduced by preincubation with the dihydropyridine agonist Bay K 8644 (20), an effect that was confirmed in experiments in which preincubation with Bay K 8644 increased several-fold both early $^{45}Ca^{2+}$ uptake and catecholamine secretion in untransfected cells and GH secretion in transfected cells. However, expression of exogenous class C $\alpha 1$ subunits did not significantly increase the ability of Bay K 8644 to enhance secretion between 0 and 30 sec (Fig. 6A). The exogenous L-type Ca^{2+} channels may respond differently than the endogenous channels by not opening rapidly after preincubation with Bay K 8644, perhaps because of differences between the interactions of the exogenous and endogenous class C $\alpha 1$ subunits with other subunits needed to form a functional channel. It should be noted that Bay K 8644 did increase GH secretion due to the extra channels during the latest time interval investigated (5–15 min). This may reflect a feature shared with endogenous L-type channels since Bay K8644 prevents inactivation of endogenous L-type channels in chromaffin cells (20).

In summary, the channels induced by transfection with the mouse class C $\alpha 1$ subunit alter secretion in a manner consistent with the delayed opening of the channels, a characteristic that is strikingly similar to the behavior of endogenous L-type channels. However, the inability of Bay K 8644 to decrease the latency in channel function in secretion distinguishes the exogenous from the endogenous L-type channel.

An alternative explanation for the delay in the enhancement of secretion by the transfected channels (even in the presence of Bay K 8644) is that influx through endogenous N-, P-, and L-type channels couples more efficiently to secretion than the transfected channels (39, 40). Recent experiments suggest that most Ca^{2+} channels that couple to secretion in bovine chromaffin are not physically associated with

release sites (41). However, it is possible that endogenous Ca^{2+} channels are localized closer to release sites than transfected channels, perhaps because the endogenous channels prevent the formation of transfected channels close to release sites. Although the new channels may open rapidly, Ca^{2+} influx through them may not couple efficiently to the trigger mechanism. In this case, the transfected channels could have another effect. In bovine chromaffin cells, we (42, 43) and others (44, 45) have identified a step in addition to the final triggering of secretion where Ca^{2+} acts in the exocytotic pathway. A similar step has been identified in pituitary melanotrophs (46, 47). Ca^{2+} greatly enhances the ability of ATP to prime secretory granules to undergo exocytosis (42, 43). This may be occurring not at the plasma membrane but instead deeper within the cell and may be less localized. Thus, it is possible that Ca^{2+} entry through the exogenous channels enhances the later rates of secretion, in part through the enhancement of priming processes within the cell. Because the priming process requires time, enhanced secretion due to priming may not be evident as an enhancement of the initial rates of secretion.

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