Linopirdine Modulates Calcium Signaling and Stimulus-Secretion Coupling in Adrenal Chromaffin Cells by Targeting M-Type K⁺ Channels and Nicotinic Acetylcholine Receptors

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ABSTRACT

Adrenal chromaffin cells synthesize and release catecholamines and several other transmitters that play important physiological roles in the coordinated response to stress or danger. The main trigger for secretion is acetylcholine (ACh) released from splanchnic nerve terminals that activates nicotinic ACh receptors (nAChRs) on the chromaffin cells, causing membrane depolarization and Ca²⁺ entry primarily through voltage-gated Ca²⁺ channels (Ca-channels). G protein-coupled receptors (GPCRs) can also trigger secretion, and it has been suggested that closure of M-type K⁺ channels might contribute to this process. However, GPCRs have multiple effects on calcium signaling and secretion, including release of intracellular Ca²⁺ stores, activation of second messenger pathways and kinases, and Ca²⁺ entry through store/receptor-operated channels. Hence, the effects of M-channel closure on [Ca²⁺]i signaling and transmitter release remain unclear. We have investigated the effects of linopirdine, a relatively selective blocker of M-channels, on stimulus-secretion coupling in chromaffin cells. Linopirdine produced a small increase in [Ca²⁺]i, in ~63% of cells because of influx through Ca-channels. However, this was not sufficient to promote catecholamine release. We also show that linopirdine reduced cholinergic-stimulated increases in [Ca²⁺]i, and secretion, primarily through potent block of nAChRs and a subtle effect on Ca²⁺ entry via Ca-channels. Hence, our data support the hypothesis that M-channels help control the excitability of chromaffin cells, but additional pathways need to be recruited by GPCRs to trigger catecholamine release. Furthermore, linopirdine potentially targets nAChRs to modulate stimulus-secretion coupling in adrenal chromaffin cells.

Adrenal chromaffin cells synthesize and release catecholamines and several other biologically active transmitters that play important physiological roles in the coordinated response to stress or danger (e.g., the “fight or flight” response). Furthermore, dysfunction in the synthesis and/or release of these transmitters might contribute to various diseases, including hypertension (Floras, 2003; Mahapatra et al., 2005). The main trigger for secretion is acetylcholine (ACh) released from splanchnic nerve terminals that activates nicotinic ACh receptors (nAChRs) on the chromaffin cells, causing membrane depolarization and Ca²⁺ entry through voltage-gated Ca²⁺ channels (Boader et al., 1987). Calcium entry directly through nAChRs might also contribute. Secretion can also be stimulated by activation of several G protein-coupled receptors (GPCRs), including muscarinic ACh receptors. Although muscarinic (and other GPCRs) can elevate free cytosolic calcium concentration ([Ca²⁺]i) by several pathways, including release from intracellular stores (Cheek and Burgoyne, 1985; Kao and Schneider, 1985), extracellular Ca²⁺ influx via voltage-gated Ca²⁺ channels and perhaps transient receptor potential/calcium-release-activated calcium current channels seems to be particularly important for triggering secretion (Zerbes et al., 1998; Fomina and Nowycky, 1999; Obukhov and Nowycky, 2002; Marley, 2003).

A recent study has shown that chromaffin cells exhibit a small M-type K⁺ channel current (M-current) (Wallace et al., 2002). M-current was first described in sympathetic neurons (Brown and Adams, 1980) where it is inhibited by several GPCRs, including muscarinic ACh receptors. It is thought that the M-channels in sympathetic neurons are heteromeric, comprised of KCNQ2 and KCNQ3 subunits (Wang et al., 1998). However, monomeric KCNQ2 or KCNQ3 channels...
also produce M-like currents, and KCNQ5 and erg1 subunits might also contribute in different cells types (Lerche et al., 2000; Schroeder et al., 2000; Selyanko et al., 2002). M-channels are noninactivating, open near to the resting membrane potential of cells, and play a key role in controlling neuronal excitability. Closure of the M-channels by GPCR activation or drugs will tend to depolarize the membrane potential, leading to increased excitability and firing of action potentials (Brown and Yu, 2000; Jentsch, 2000; Robbins, 2001).

It has been shown that block of the small M-current in chromaffin cells can lead to a small membrane depolarization and increased action potential firing that might underlie the Ca\(^{2+}\) entry and catecholamine release stimulated by GPCRs (Wallace et al., 2002). However, the contribution of M-channels to [Ca\(^{2+}\)]i signaling and catecholamine release in isolation from the other pathways activated by GPCRs remains unclear.

In this study, we have used fluorescent imaging of [Ca\(^{2+}\)]i, patch-clamp electrophysiology, and high-performance liquid chromatography methods to investigate the effects of linopirdine, a relatively selective blocker of M-channels (Aiken et al., 1995; Lamas et al., 1997), on stimulus-secretion coupling in chromaffin cells. Linopirdine increases release of ACh and norepinephrine in some neuronal populations and has been investigated as a putative cognition enhancer (Aiken et al., 1995; Lamas et al., 1997), on stimulus-secretion coupling including nicotinic ACh receptors (nAChRs) (Lamas et al., 1997; Schnee and Brown, 1998; Kristufek et al., 1999). Our data show that when linopirdine was applied under resting conditions, it led to a small increase in [Ca\(^{2+}\)]i, in ~63% of chromaffin cells. This was because of influx of extracellular Ca\(^{2+}\) through voltage-gated Ca\(^{2+}\) channels, consistent with block of M-channels leading to membrane depolarization. However, the increase in [Ca\(^{2+}\)]i, caused by M-channel block with linopirdine was not sufficient to promote catecholamine release. We also show that linopirdine reduced rather than augmented cholinergic-stimulated catecholamine release. This was primarily because of potent block of nAChRs at concentrations commonly used to block M-channels (<10 \(\mu\)M). At slightly higher concentrations (30 \(\mu\)M), linopirdine also produced a small reduction in Ca\(^{2+}\) entry and catecholamine release evoked by KCl, suggesting a subtle effect on voltage-gated Ca\(^{2+}\) channels.

Overall, our data provide new insight into the role of M-channels in chromaffin cells and into the mechanisms of action of linopirdine, which not only targets M-channels but also nAChRs to modulate stimulus-secretion coupling in adrenal chromaffin cells.

**Materials and Methods**

**Cell Culture.** Adult bovine adrenal glands were obtained from a local slaughterhouse, and chromaffin cells were prepared by digestion with collagenase followed by density gradient centrifugation based on previously published protocols (Fenwick et al., 1978; Greenberg and Zinder, 1982). The cells were plated onto coverslips coated with collagen (at a density of 0.3–0.4 \(\times\) 10⁶ cells/ml for [Ca\(^{2+}\)], measurements or ~0.2 \(\times\) 10⁶ cells/ml for patch-clamp recordings). For secretion studies, cells were plated in 24-well tissue culture plates coated with collagen at a density of ~0.5 \(\times\) 10⁶ cells/well. Cells were maintained in an incubator at 37°C in an atmosphere of 95% air and 5% CO\(_2\) with a relative humidity of ~90%. Fibroblasts were effectively suppressed with cytosine-arabinoside (10 \(\mu\)M) (Sigma-Aldrich, St. Louis, MO), leaving relatively pure chromaffin cell cultures. One-half of the culture medium was exchanged every day. This medium consisted of Dulbecco’s modified Eagle’s medium/Ham’s F-12 (1:1) supplemented with 10% fetal bovine serum, 2 \(\mu\)M glutamine, 100 unit ml\(^{-1}\)100 pg ml\(^{-1}\) penicillin/streptomycin, 10 \(\mu\)M cytosine arabinoside, and 10 \(\mu\)M 5-fluorodeoxyuridine. Unless otherwise noted, all tissue culture reagents were from Invitrogen (Carlsbad, CA) apart from FBS, which was from Hyclone Laboratories (Logan, UT). All experiments were performed 1 to 5 days following cell isolation.

**[Ca\(^{2+}\)]i.** [Ca\(^{2+}\)]i was measured in cells loaded with the fluorescent Ca\(^{2+}\) indicator Fura-2 or its lower affinity derivative Fura-4F (Molecular Probes, Eugene, OR). Cells were washed twice with HBES-buffered Hanks’ balanced salt solution (HBSS) and incubated for 30 to 45 min with 2 \(\mu\)M Fura-2-AM or Fura-4F-AM at 37°C. Cells were then washed in Fura-free solution for 30 to 60 min before recording. For recording, the coverslip with the cells attached was transferred to a recording chamber and mounted on the stage of Nikon TE2000 fluorescence microscope. The recording chamber had a volume of ~300 to 400 \(\mu\)l and was continually perfused with fresh solution (see below for composition) from gravity-fed reservoirs at a flow rate of ~4 ml/min. An InCyt IM2 fluorescence imaging system (Intracellular Imaging Inc., Cincinnati, OH) was used to monitor [Ca\(^{2+}\)]i. Cells were alternately excited at wavelengths of 340 and 380 nm, and emission at 510 nm was detected using a PixelFly digital camera (Intracellular Imaging Inc., Cincinnati, OH). Usually, 10 to 20 individual cells were selected on each coverslip, and images were captured and processed using the InCyt IM2 software. Backgrounds were subtracted from the individual wavelengths, and the ratio of fluorescence at 340 nm/380 nm was determined. Ratios were collected every 2 s throughout the experiment and converted to [Ca\(^{2+}\)]i using an in vitro calibration curve. The calibration curve was generated by adding 50 \(\mu\)M Fura-2 (or Fura-4F) pentapotassium salt to solutions that contained known concentrations of Ca\(^{2+}\) (0–1350 nM). Data analysis was performed using OriginPro software (OriginLab Corp., Northampton, MA).

To determine whether a cell was considered to respond to linopirdine application with a rise in [Ca\(^{2+}\)], we set an arbitrary threshold value of 20 nM. This value was arrived at by analyzing [Ca\(^{2+}\)], in ~150 individual cells under resting conditions for 5 min. For each cell, we calculated the mean and standard deviation of the mean resting [Ca\(^{2+}\)], over this 5-min period. The threshold was set to ~3 times the standard deviation, which on average was ~7 nM. Hence, when calculating the proportion of cells that responded to linopirdine, those cells with a change in [Ca\(^{2+}\)] >20 nM were “responders”, and those cells with changes in [Ca\(^{2+}\)] <20 nM were considered nonresponders. Mean resting [Ca\(^{2+}\)], was 158 ± 3 nM. Approximately 6% of cells had resting [Ca\(^{2+}\)], >300 nM and were excluded from analysis.

The composition of the NaCl-based extracellular medium was 145 mM NaCl, 2 mM KCl, 2 mM CaCl\(_2\), 1 mM MgCl\(_2\), 10 mM glucose, and 10 mM HEPES, pH 7.3, adjusted with NaOH and ~305 mM. In some experiments, cells were stimulated with a solution containing high KCl to directly depolarize the cell membrane; for this purpose, the concentration of KCl was increased to 60 mM and NaCl was reduced accordingly to maintain osmolarity. For stimulation with carbachol, preliminary experiments (not shown) indicated the EC\(_{50}\) was approximately 30 \(\mu\)M. Use of 30 \(\mu\)M carbachol enabled multiple reproducible responses to be obtained from the same cells. We also used 100 \(\mu\)M carbachol in some experiments to produce a near maximal response and to mimic the stimulation in the catecholamine release experiments.

**Catecholamine Release Experiments.** To determine whether linopirdine caused catecholamine release, chromaffin cells were plated in 24-well plates at a density of ~0.5 \(\times\) 10⁶ cells/well. The cells
were washed twice with HEPES-buffered HBSS and then incubated in this solution for 30 min at 37°C. After this 30-min "preincubation," the medium was removed and 600 µl of NaCl-based extracellular solution with or without 30 mM linopirdine was added to the cells for 10 min at 37°C. After the 10-min "stimulation" period, the cells were placed on ice, and the medium was collected and added to an equal volume of ice-cold 0.4 M perchloric acid. Perchloric acid was also added to the tissue culture wells containing the cells to extract the nonreleased catecholamines. The catecholamine content of the samples was determined in the Neurochemistry Core of the Center for Molecular Neuroscience (Vanderbilt University, Nashville, TN). In brief, catecholamines were determined by a specific HPLC assay using an Antec Decade (oxidation, 0.7; GBC Separations Inc., Hubbardstown, MA) electrochemical detector. Samples (20 µl) were injected using a Waters 717+ autosampler onto a Waters Nova-Pak C18 HPLC column. Biogenic amines were eluted with a mobile phase consisting of 89.5% 0.1 M trichloroacetic acid, 10−2 M sodium acetate, 10−4 M EDTA, and 10.5% methanol, pH 3.8. The method was delivered at 0.7 µl/min using a Waters 515 HPLC pump. HPLC control and data acquisition were managed by Millennium32 software. The amount of catecholamine released into the bathing medium during the 10-min incubation was expressed as a percentage of the total catecholamine content for that dish of cells. All treatments were performed in duplicate and repeated at least 3 to 4 independent experiments performed on different cell preparations. Basal release, determined by incubating cells with the NaCl-based extracellular medium, was typically <1% of total content and is shown for all data sets.

In other experiments the effect of linopirdine on catecholamine release stimulated by 100 µM carbachol or 60 mM KCl was determined. The cells were preincubated for a total of 30 min. The first 20 min of the preincubation was with NaCl-based medium and then an additional 10-min preincubation was with either NaCl-based medium (controls) or NaCl-based medium containing linopirdine. Cells were stimulated with the secretagogue for 10 min in the absence or presence of linopirdine as appropriate. Again, all treatments were performed in duplicate on multiple cell preparations.

Patch-Clamp Electrophysiology. Cells were plated onto collagen-coated glass coverslips at 0.1 to 0.2 × 10⁶ cells/mL and patch-clamped in the standard whole-cell recording configuration using an Axopatch 200B amplifier (Axon Instruments, Union City, CA) and custom software written in AxD BASIC, or Windows-based programs written in the Visual Basic environment, kindly provided by Dr. Aaron Fox (University of Chicago, Chicago, IL). Data were filtered at 2 kHz and sampled every 20 to 100 µs. Analysis was performed using custom-written programs and OriginPro software. Electrodes were pulled from microhemocrit capillary tubes (Drummond Scientific, Broomall, PA) and coated with Sylgard (Dow Corning, Midland, MI). After fire polishing, electrodes had resistances of ~2 MΩ. The patch pipette solution consisted of 110 mM CsCl, 4 mM MgCl₂, 20 mM HEPES, 10 mM EDTA, and 14 mM creatine phosphate, pH 7.3, osmolality approximately 310 to 315 mOsm. For recording inward nicotinic currents elicited by carbachol, the extracellular solution was the same as that used for the [Ca²⁺]i imaging experiments. For recording voltage-gated Ca²⁺ channel currents (I_Ca), extracellular [Ca²⁺] was increased to 10 mM. Series resistance was partially compensated using the Axopatch circuitry, and data for I_Ca were subjected to linear leak and capacitance subtraction. During experiments, cells were voltage-clamped at a holding potential of ~80 mV to minimize any steady-state inactivation of I_Ca, and the recording bath (total volume ~250–300 µl) was continually washed with fresh extracellular medium at a rate of ~4 µl/min from gravity-fed reservoirs.

Nicotinic ACh receptor currents were activated by bath perfusion for 30 s with 30 µM carbachol. Use of 30 µM carbachol enabled multiple reproducible responses to be obtained from the same cell. The delay in current activation was because of the "dead space" in the perfusion system. The amplitude of the sustained inward current activated by carbachol was determined by calculating the mean current amplitude over the 5-s period starting 20 s after carbachol application. The mean current amplitude over this 5-s period was determined for each cell and then data were pooled. Data are reported as mean ± S.E.M., and statistical significance was determined using paired or independent Student's t test or analysis of variance as appropriate.

Drugs and Reagents. The AM-esters and pentapotassium salts of Fura-2 and Fura-4F, and a Ca²⁺ calibration set containing buffers with known Ca²⁺ concentrations, were from Molecular Probes. The AM-esters were prepared as 1 mM stock in dimethyl sulfoxide, and aliquots were frozen for up to 1 to 2 weeks. The salts were prepared as aqueous stocks and stored frozen or at 4°C. For loading cells, the AM-ester was diluted into HEPES-buffered HBSS containing 1 mg/ml bovine serum albumin. The final concentration was 2 µM. Linopirdine and Xe991 were purchased from Tocris Cookson Inc. (Ellisville, MO) and prepared as a stock solution (100 mM) in sterile H₂O, and aliquots were frozen until use. Carbachol was purchased from Sigma-Aldrich, and stocks prepared in sterile H₂O and kept refrigerated. ω-Conotoxin GVIA and ω-agatoxin IVA were from Alomone Labs (Jerusalem, Israel). Stocks (100–300 µM) were prepared in ethanol and stored at ~20°C protected from light until use.

Results

Linopirdine Stimulates Ca²⁺ Influx via Voltage-Gated Ca²⁺ Channels but Does Not Promote Catecholamine Secretion. It has been reported recently that adrenal chromaffin cells exhibit a small M-type K⁺ current (Wallace et al., 2002). Block of M-current can produce a small membrane depolarization and lead to action potential firing, but the effects of linopirdine/M-current blockade on [Ca²⁺]i signaling and catecholamine secretion remain unclear. We used fluorescent imaging to measure [Ca²⁺]i, in individual chromaffin cells loaded with Fura-2. Cells were continuously perfused with fresh extracellular recording solution and exposed to linopirdine (3-min applications) at various concentrations (1–300 µM) (Fig. 1A). Linopirdine produced a small dose-dependent increase in [Ca²⁺]i, that was maintained for the duration of drug application. After washout of linopirdine, [Ca²⁺]i returned to baseline. The EC₅₀ was estimated to be ~29 µM (Fig. 1B). For the remaining experiments, we used linopirdine at concentrations of ≤30 µM. Approximately 63% (233 of 367) of the cells responded to 30 µM linopirdine with a mean increase in [Ca²⁺]i of 111 ± 5 nM (n = 233). In the remaining cells (nonresponders), the change in [Ca²⁺]i produced by linopirdine was 4 ± 0.5 nM (n = 134). We also tested the effects of Xe991, an analog of linopirdine that is also a potent M-channel blocker (Wang et al., 1998). The same cells were exposed to Xe991 (30 µM), linopirdine (30 µM), and to both drugs in combination. Xe991 produced an increase in [Ca²⁺]i, of 101 ± 17 nM. In the same cells, linopirdine produced an increase of 127 ± 27 nM, but there was no significant increase in [Ca²⁺]i when both drugs were applied simultaneously (148 ± 23 nM; n = 29; p = 0.43). Hence, the effects were not additive, suggesting the drugs occlude one another and act at the same target (presumably M-channels).

The increase in [Ca²⁺]i, produced by linopirdine was abolished when cells were bathed in Ca²⁺-free extracellular medium. Cells were initially bathed in standard extracellular solution (containing 2 mM Ca²⁺) and exposed to 30 µM
linopirdine acts by blocking M-channels to cause membrane depolarization and opening of voltage-gated Ca\(^{2+}\) channels.

Bovine chromaffin cells express L-type, N-type, and P/Q-type Ca\(^{2+}\) channels and also a smaller blocker-resistant I\(_{Ca}\) (Albillos et al., 1993; Artalejo et al., 1994; Currie and Fox, 1996). Selective blockers of voltage-gated Ca\(^{2+}\) channels reduced the response to linopirdine (Fig. 1D). Nitrendipine (10 \(\mu\)M), a selective dihydropyridine antagonist of L-type Ca\(^{2+}\) channels, reduced the response to linopirdine by \(\sim 52\%\).

The data mentioned above (Fig. 1A–D) show that linopirdine caused a modest (100–150 nM) increase in [Ca\(^{2+}\)]\(_i\), in approximately two-thirds of chromaffin cells. The increase in [Ca\(^{2+}\)]\(_i\) was sustained for as long as linopirdine was present, even for up to 10 min (not shown). To determine whether linopirdine could stimulate catecholamine release, cells were plated at \(-0.5 \times 10^6\) cells/well in 24-well tissue culture plates. Cells were washed and then incubated with extracellular medium for 10 min at 37°C to determine basal catecholamine secretion.

Linopirdine was determined using HPLC and expressed as a percentage of the total content of the cells in that dish. In parallel experiments, dishes of cells prepared on the same day were incubated for 10 min at 37°C with extracellular solution containing 30 \(\mu\)M linopirdine. Linopirdine had no effect on epinephrine or norepinephrine release (Fig. 1, E and F).

Linopirdine Reduced Cholinergic-Evoked Catecholamine Release. Although the increase in [Ca\(^{2+}\)]\(_i\), produced by linopirdine was small and not sufficient to trigger catecholamine release per se, we postulated that it might summate with, and augment the Ca\(^{2+}\) influx and catecholamine release triggered by known secretagogues. Furthermore, small sustained increases in [Ca\(^{2+}\)]\(_i\), might mobilize secretory vesicles and increase the size of the release-ready pool of vesicles, thereby increasing the secretory response to subsequent stimuli (von Ruden and Neher, 1993; Smith et al., 1998; Pan and Fox, 2000). In situ, chromaffin cells in the adrenal medulla are innervated by splanchnic nerve fibers that release ACh. The released ACh activates both nicotinic and muscarinic ACh receptors to elevate [Ca\(^{2+}\)]\(_i\), and trigger secretion.

Hence, we tested the effects of linopirdine on catecholamine release produced by carbachol, a nonselective cholinergic agonist that is resistant to cholinesterase activity.

Epinephrine and norepinephrine release were assessed using HPLC and expressed as a percentage of total cellular content. Cells were stimulated for 10 min with 100 \(\mu\)M carbachol in the absence or presence of linopirdine (3 or 30 \(\mu\)M). Basal secretion was also determined by incubating cells with NaCl-based extracellular medium (no carbachol). Cells were exposed to NaCl-based medium with or without linopirdine as appropriate for 10 min before stimulation with carbachol.

As shown in Fig. 2, carbachol produced a robust increase in epinephrine release (above basal), and this was significantly reduced by linopirdine at both 3 \(\mu\)M (\(-17\%\) reduction; \(n = 6\); \(p < 0.005\)) and 30 \(\mu\)M (\(-54\%\) reduction; \(n = 6\); \(p < 0.001\)).
Although as described for A, except secretion was stimulated by 60 mM KCl. Linopirdine reduced secretion by 2B). Similar effects were seen for K

Linopirdine Blocks Cholinergic-Mediated Ca\textsuperscript{2+} Influx. Cholinergic stimulation produces catecholamine release primarily via activation of nicotinic ACh receptors that in turn depolarize the cell membrane and open voltage-gated Ca\textsuperscript{2+} channels. Chromaffin cells also express muscarinic ACh receptors that can release intracellular Ca\textsuperscript{2+} stores, although this is thought to only play a minor role in triggering catecholamine release (Cheek and Burgoyne, 1985). Muscarinic receptors are also known to inhibit M-channel activity in a number of cell types, including sympathetic neurons (Brown and Adams, 1980; for review, see Brown and Yu, 2000), and this may lead to membrane depolarization and extracellular Ca\textsuperscript{2+} influx. Under our recording conditions, the majority of the [Ca\textsuperscript{2+}] increase produced by carbachol was because of extracellular Ca\textsuperscript{2+} influx. Cells were stimulated with 30-s applications of carbachol (30 μM), first in the presence (2 mM) and then in absence of extracellular Ca\textsuperscript{2+}. Removal of extracellular Ca\textsuperscript{2+} significantly reduced the [Ca\textsuperscript{2+}] response produced by carbachol from 639 ± 22 to 82 ± 14 nM (n = 103; p < 0.00001). A third application of carbachol was applied to the same cells, again in the presence of extracellular Ca\textsuperscript{2+}. The response to carbachol recovered to 590 ± 23 nM (n = 103). Hence, under our recording conditions, ~87% of the change in [Ca\textsuperscript{2+}], produced by carbachol is because of influx of extracellular Ca\textsuperscript{2+}.

Linopirdine significantly reduced the [Ca\textsuperscript{2+}] increase produced by carbachol (Fig. 3). Initially, to parallel the stimulation used in the secretion studies (Fig. 2), we compared the response to a 10-min application of carbachol (100 μM) in control cells or in cells bathed in linopirdine (3 or 30 μM) (Fig. 3A). Linopirdine was applied for 2 min before carbachol application. The peak response to carbachol was significantly reduced by both 3 and 30 μM linopirdine (Fig. 3D). Furthermore, when linopirdine was applied during the carbachol stimulation, it produced a rapid and reversible decrease of [Ca\textsuperscript{2+}], (Fig. 3A, right).

To ensure that variability between cells or cell preparations did not contribute to the effects we were observing, the same cells were stimulated by multiple applications of 30 μM carbachol lasting 30 s, separated by an interval of 10 min (Fig. 3B). In control cells, the second response is not significantly different from the first response (Fig. 3, B, left, and C). However, the second response was significantly reduced by coapplication of linopirdine (Fig. 3, B, right, and C). This effect was readily reversible upon washout of linopirdine (Fig. 3B, right).

The effects of linopirdine on the carbachol response could be because of a direct action of the drug on the nAChRs or on voltage-gated Ca\textsuperscript{2+} channels. Alternatively, the increase in [Ca\textsuperscript{2+}], produced by linopirdine application could potentially modulate the nAChR or Ca\textsuperscript{2+} channels, perhaps by recruiting additional second messenger cascades or kinase activity. To distinguish these possibilities, we divided the cells into two groups: those cells that responded to linopirdine with an increase in [Ca\textsuperscript{2+}] (responders), and those cells that did not show an increase in [Ca\textsuperscript{2+}], in response to linopirdine (non-responders). Linopirdine blocked the response to carbachol by the same extent in both sets of cells [32 ± 4% in responders (n = 138), and 35 ± 3% in nonresponders (n = 85; p = 0.62)]. This rules out the possibility that the effect on the carbachol response is mediated by the small increase in basal [Ca\textsuperscript{2+}], produced by linopirdine. Rather, it is consistent with direct block of nicotinic ACh receptors and perhaps voltage-gated Ca\textsuperscript{2+} channels by linopirdine.

Linopirdine Blocks Nicotinic ACh Receptor Currents. Our [Ca\textsuperscript{2+}] imaging data are consistent with the idea that linopirdine, in addition to blocking M-channels, also blocks nAChRs in chromaffin cells. To directly test this possibility, we used standard whole-cell patch-clamp recording. Cells were voltage-clamped at a holding potential of −80 mV, and the bath was continuously perfused with fresh extracellular recording solution. To keep the experimental protocol consistent with that used in the [Ca\textsuperscript{2+}], imaging experiments, carbachol (30 μM) was applied to the cell by bath perfusion for 30 s. Carbachol produced a sustained inward current that returned to baseline upon washout (Fig. 4A). Under these conditions, the rapidly desensitizing component.
of the inward nicotinic current will not be detectable. The inward current we observe is because of the sustained activation of the nondesensitizing nACh receptors found in chromaffin cells that cause the sustained membrane depolarization, Ca\textsuperscript{2+} influx, and catecholamine release during prolonged carbachol application. After 5-min recovery, the cell was perfused with linopirdine (3 or 30 μM) for 2 min before a second application of carbachol. Linopirdine itself did not have an effect on the cells (not shown), but it did significantly block the inward current evoked by carbachol.

![Image](44x375 to 296x737)

**Fig. 3.** Linopirdine reduced the increase in \([\text{Ca}^{2+}]_{i}\), produced by cholinergic stimulation. \([\text{Ca}^{2+}]_{i}\), was measured in cells loaded with Fura-4F. The bath was continually perfused with fresh extracellular medium, and drugs were applied at the times indicated by the horizontal bars. A, representative experiments showing the \([\text{Ca}^{2+}]_{i}\) response to 10-min applications of 100 μM carbachol. Left, control cells (mean response of 23 cells); middle panel, cells pre-exposed to 30 μM linopirdine (mean response of 11 cells); and right panel, 30 μM linopirdine applied during the carbachol response (mean response of 14 cells). B, representative experiments showing the \([\text{Ca}^{2+}]_{i}\) response to multiple applications of carbachol (30 μM (30-s applications separated by 10 min). Left, two applications of carbachol produced identical responses in control cells (mean response of 19 cells); and right, linopirdine (30 μM) reversibly reduced the response to carbachol (mean response of 24 cells). C, mean data from multiple experiments like that shown in B. The second response was normalized to the first response within the same cell. The second response in the presence of linopirdine (3 μM, n = 67 or 30 μM, n = 223) was significantly reduced compared with the second response in control cells (n = 100) (\(\ast\), \(p < 0.0001\); **, \(p < 0.00001\)). D, mean data from multiple experiments like that shown in A (10-min applications of carbachol). Carbachol was applied to control cells (n = 93) or to cells in the presence of 3 μM (n = 73) or 30 μM (n = 68) linopirdine (\(\ast\), \(p < 0.0001\)).

![Image](326x459 to 542x737)

**Fig. 4.** Linopirdine blocked nicotinic ACh receptor currents. Cells were voltage-clamped in the whole-cell recording configuration and continuously washed with fresh extracellular medium. Carbachol was applied by bath perfusion for 30 s as indicated. A, three currents recorded from the same cell showing reversible block by linopirdine (3 μM). B, mean data from five cells like that shown in A (\(\ast\), \(p < 0.02\)).

![Image](1170 Dzhura et al.)

**Linopirdine Effects on \([\text{Ca}^{2+}]_{i}\) Influx through Voltage-Gated Ca\textsuperscript{2+} Channels.** In addition to blocking nAChR currents, linopirdine might also reduce the increase in \([\text{Ca}^{2+}]_{i}\), produced by carbachol by blocking Ca\textsuperscript{2+} influx through voltage-gated Ca\textsuperscript{2+} channels. Furthermore, our data show that secretion stimulated by KCl depolarization (which bypasses the nAChRs) was modestly reduced by 30 μM linopirdine (Fig. 2B). To investigate the effects of linopirdine on \(I_{\text{Ca}}\), we first used whole-cell patch-clamp recording. For these experiments, extracellular Ca\textsuperscript{2+} was raised to 10 mM. Cells were voltage-clamped at −80 mV, and inward \(I_{\text{Ca}}\) was activated by 20-ms voltage step commands to +20 mV once every 10 s. Application of 30 μM linopirdine had no effect on peak \(I_{\text{Ca}}\) (reduced by 3 ± 1.5%; \(n = 5\); \(p = 0.28\)). Furthermore, linopirdine produced no obvious shifts in the current-voltage relationship or steady-state inactivation of \(I_{\text{Ca}}\) (\(n = 5\) cells; data not shown).

To further investigate the effects of linopirdine on Ca\textsuperscript{2+} entry through voltage-gated Ca\textsuperscript{2+} channels we recorded the change in \([\text{Ca}^{2+}]_{i}\), produced by KCl (60 mM) in cells loaded
with Fura-4F. Application of KCl will directly depolarize the cells and open voltage-gated Ca\(^{2+}\) channels. Cells were continually perfused with fresh medium and stimulated by two applications of 60 mM KCl lasting 30 s (Fig. 5A). The stimuli were separated by 10 min. In control cells, the second response was significantly smaller than the first response (91 ± 1.6% of first response; \(n = 55\); \(p < 0.001\)) (Fig. 5B). In other cells, the second application of KCl was in the presence of 30 \(\mu\)M linopirdine (Fig. 5A). Similar to control cells, the second response was smaller than the first response (84 ± 3.5%; \(n = 59\); \(p < 0.004\)). However, there was no significant difference in the second responses compared between control cells and linopirdine-treated cells (\(p = 0.11\)) (Fig. 5B).

In other experiments, cells were stimulated with 10-min applications of 60 mM KCl to mimic the conditions used in the secretion assay. Cells exposed to linopirdine before and during the application of KCl were compared with control cells (no linopirdine). The peak change in [Ca\(^{2+}\)]\(_i\) was not significantly different from that of control cells (−8% decrease from 3002 ± 103 nM, \(n = 66\) to 2764 ± 97 nM, \(n = 76\); \(p = 0.10\)). However, linopirdine did have an effect on the time course of the [Ca\(^{2+}\)]\(_i\) increase. During the 10-min application of KCl, [Ca\(^{2+}\)]\(_i\), declined almost back to baseline but was maintained at an elevated plateau (Fig. 5, C and D). In control cells, this plateau level was 11 ± 0.5% of the peak response (\(n = 66\)). This maintained plateau was significantly lower in the presence of linopirdine (7 ± 0.5% of peak; \(n = 76\); \(p < 0.0001\)). Linopirdine also produced a small, reversible block of the [Ca\(^{2+}\)]\(_i\) response when applied during the KCl stimulation (Fig. 5D). The KCl was applied by bath perfusion for 10 min. After the first 2 min of the stimulation, linopirdine (30 \(\mu\)M) was coapplied with the KCl for 3 min and then washed out again for the remaining 5 min of the stimulation (Fig. 5D). We compared the amplitude of the [Ca\(^{2+}\)]\(_i\) change immediately before washout of linopirdine (4 min and 55 s into the KCl stimulation) to the amplitude immediately after washout of linopirdine (1 min after washout). Washout of linopirdine increased [Ca\(^{2+}\)]\(_i\), from 306 ± 10 to 451 ± 13 nM (\(n = 101\); \(p < 0.00001\)), indicating that the presence of linopirdine significantly reduced KCl-stimulated Ca\(^{2+}\) entry.

Overall, these data suggest that linopirdine has subtle effects on Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels. Although there was little effect on Ca\(^{2+}\) influx elicited by brief stimuli, with sustained depolarization lasting several

![Fig. 5](image-url)
noninactivating M-type K⁺ channels are active close to the resting membrane potential, so they play a role in controlling neuronal excitability (for reviews, see Brown and Yu, 2000; Jentsch, 2000; Robbins, 2001). Linopirdine is a relatively selective blocker of M-channels that has been shown to enhance ACh and norepinephrine release from certain neurons (Aiken et al., 1995, 1996; Lamas et al., 1997; Kristufek et al., 1999). It has been postulated that this enhancement of neurotransmitter release might be because of blockade of somatic M-type K⁺ channels, leading to increased excitability/action potential firing. In this study, we have used linopirdine as a pharmacological tool to investigate the role of M-channels in neuroendocrine chromaffin cells. We have also investigated the broader effects of linopirdine on stimulus-secretion coupling and show that it potently blocks cholinergic-mediated Ca²⁺ entry and catecholamine release, primarily through blockade of nicotinic ACh receptors.

Chromaffin cells express a small M-like K⁺ channel current, and it has been suggested that closure of these channels might contribute to the stimulation of Ca²⁺ influx and catecholamine release by GPCRs (Wallace et al., 2002). However, GPCRs have multiple effects on Ca²⁺ dynamics, including release of intracellular Ca²⁺ stores, activation of second messenger pathways and kinases, and perhaps Ca²⁺ entry through store/receptor-operated channels (such as transient receptor potential channels) (Cheek and Burgoyne, 1985; Zerbes et al., 1998; Fomina and Nowycky, 1999; Obukhov and Nowycky, 2002; Marley, 2003). The effects of M-channel closure per se on [Ca²⁺]i, signaling and transmitter release remain unknown.

The data we present in this study support the hypothesis that M-channels contribute to the control of resting membrane potential in chromaffin cells, consistent with the data of Wallace et al. (2002). Approximately 63% of chromaffin cells responded to linopirdine with a small rise in [Ca²⁺]i (~100–150 nM) that was maintained for the duration of the drug application. The response was abolished by removal of extracellular Ca²⁺, and selective blockers of voltage-gated Ca²⁺ channels reduced the response by ~82%. This is consistent with M-channel blockade causing membrane depolarization and opening of voltage-gated Ca²⁺ channels. Approximately one-third of the cells did not respond to linopirdine with a [Ca²⁺]i increase. It might be that these cells did not express functional M-channels. Alternatively, it might be that these cells do express M-channels but that the extent of the membrane depolarization produced by block of these channels was not sufficient to reach threshold for action potential firing and/or opening of voltage-gated Ca²⁺ channels.

The EC₅₀ for the increase in [Ca²⁺]i produced by linopirdine was ~29 μM, similar to the concentrations of linopirdine reported to block the M-channel current in chromaffin cells (~45% block by 10 μM and ~95% by 100 μM) (Wallace et al., 2002). These values are slightly higher than those reported (4–10 μM) for block of M-current in sympathetic neurons or heterologously expressed KCNQ2/3 K⁺ channel currents (Lamas et al., 1997; Schnee and Brown, 1998; Robbins, 2001). This might be because of subtle differences in the molecular composition of the M-channels in chromaffin cells. Although it is thought that heteromeric channels comprised of KCNQ2/3 K⁺ channel subunits underlie M-current in sympathetic neurons, monomeric KCNQ2, -3, or -5 channels also produce M-like currents, and erg1 subunits might also contribute in some cell types (Wang et al., 1998; Lerche et al., 2000; Schroeder et al., 2000; Selyanko et al., 2002). Further investigation will be needed to determine which of these subunits are expressed in chromaffin cells. It is also possible that higher concentrations of linopirdine might block other K⁺ channel types (Lamas et al., 1997; Schnee and Brown, 1998). To minimize this possibility, we used linopirdine concentrations ≤30 μM.

Although linopirdine did produce a small [Ca²⁺]i increase (~100–150 nM), our data show that this was not sufficient to promote catecholamine release (Fig. 1, E and F). Even with 10-min incubations of linopirdine, the release of epinephrine and norepinephrine were not increased above basal levels. This does not rule out the involvement of M-channel closure in the secretory response to GPCR agonists (such as histamine), but it does suggest that M-channel closure by itself is not sufficient to trigger release. Rather, summation with other GPCR-mediated signaling pathways that elevate [Ca²⁺]i and/or second messenger cascades is required.

We also investigated the effect of linopirdine on secretion triggered by cholinergic agonists or by direct membrane depolarization with 60 mM KCl. We postulated that although the [Ca²⁺]i increase produced by linopirdine was not sufficient to produce secretion per se, it might augment the [Ca²⁺]i increase and secretion produced by other stimuli. We chose to use carbachol, a nonspecific cholinergic agonist resistant to cholinesterase activity, because in the intact adrenal gland, ACh release from preganglionic sympathetic (splanchnic) nerve terminals is the primary physiological trigger for catecholamine release, acting primarily at nicotinic but also at muscarinic receptors. Linopirdine (both 3 and 30 μM) significantly reduced the rise in [Ca²⁺]i and catecholamine secretion produced by carbachol. Patch-clamp recording showed that nicotinic ACh receptor currents elicited by carbachol were potently blocked by linopirdine (56% block by 3 μM, 95% block by 30 μM). Hence, in chromaffin cells, the dominant effect of linopirdine on stimulus-secretion coupling is mediated not through block of M-channels but through block of nicotinic ACh receptors.

Chromaffin cells express multiple subtypes of nAChR. In addition to channels comprised of α5β4* subunits, there is also evidence for contributions of α9 and α7 subunits (Garcia-Guzman et al., 1995; Campos-Caro et al., 1997; Lopez et al., 1998; Di Angelantonio et al., 2003). It is important to note that our recording conditions (bath application of carbachol for 30 s) do not enable the detection of currents carried by rapidly desensitizing α7-containing channels. Further studies making use of fast, brief agonist applications are required to fully address the effects of linopirdine on chromaffin cell nAChR currents. It has been reported that linopirdine can block α9/α10-containing nAChR in cochlear hair cells (Gomez-Casati et al., 2004) and the nAChR in sympathetic neurons (Lamas et al., 1997; Schnee and Brown, 1998;
Krstufek et al., 1999). Our data show that the nonselectivity of nAChR current in chromaffin cells, likely mediated by α3β4 receptors, is also potently blocked by linopirdine. It is interesting to note the extent by which the nAChR currents were blocked by linopirdine is much greater than the effect on [Ca²⁺]i. This could be because of the contribution of Ca²⁺ released from intracellular stores (because of concomitant muscarinic receptor activation) or to Ca²⁺ influx mediated by other pathways, such as store/receptor-operated channels. It is also possible that there is a significant reserve of nAChRs to ensure reliable membrane depolarization upon cholinergic stimulation, even when a large percentage of the channels are blocked (or desensitized).

Our data also showed that secretion evoked by direct membrane depolarization (using 60 mM KCl) was reduced (~30%) by 30 μM linopirdine, whereas 3 μM linopirdine did not have any effect. Under these conditions, the nAChRs are bypassed, suggesting that linopirdine might also have direct effects on Ca²⁺ entry through voltage-gated Ca²⁺ channels. Patch clamp recording revealed little effect of 30 μM linopirdine on Ica elicited by brief step deparolizations. However, linopirdine produced a small but significant reduction in the sustained [Ca²⁺]i increase produced by a 10-min application of KCl. Why might linopirdine have little effect on the peak change in [Ca²⁺]i, or on Ica, activated by brief step depolarizations but produce a significant reduction during sustained KCl stimulation? One possibility is that linopirdine selectively blocks one subtype of Ica. During brief depolarizations, Ica is mediated predominantly by N- and P/Q-type Ca²⁺ channels, with a small contribution from L-type Ca²⁺ channels (Currie and Fox, 1996). However, the sustained Ca²⁺ entry during prolonged KCl application is mediated mostly by L-type channels, because the N- and P/Q-type channels undergo greater inactivation (Burgoyne and Cheek, 1985; Villarroya et al., 1999). It is also possible that linopirdine is an open channel blocker and that its effects are accentuated under conditions when most of the channels are already open. Future experiments will be needed to distinguish these possibilities and to clarify the mechanism of action of linopirdine on Ca²⁺ channels.

Overall, we show that M-channels help control the resting membrane potential of chromaffin cells and that block of these channels causes a modest excitation and [Ca²⁺]i increase. However, the [Ca²⁺]i increase produced by M-channel blockade is not sufficient to stimulate catecholamine secretion. We also show that linopirdine reduces cholinergic-mediated Ca²⁺ entry and secretion, predominantly through block of nAChR but also by a subtle effect on Ca²⁺ entry through voltage-gated Ca²⁺ channels. Block of presynaptic as well as postsynaptic neuronal nAChR will likely mediate at least some of the behavioral effects of linopirdine and as well as postsynaptic neuronal nAChR will likely mediate a block of nAChR but also by a subtle effect on Ca²⁺ influx.

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References


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