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Research report

# Progesterone regulation of catecholamine secretion from chromaffin cells

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**Abstract**

Stress stimulates the adrenal medulla to rapidly secrete catecholamines (CAs), and the adrenal cortex to release progesterone (PROG), which may locally regulate stress-induced CA release. We used bovine chromaffin cells to investigate the effects of PROG on CA secretion. PROG dose-dependently inhibited CA secretion induced by nicotinic acetylcholine receptor (nAChR) agonist 1,1-dimethyl-4-phenlypiperazinium iodide (DMPP) up to 77%. Pre-incubation with PROG up to 1 h increased this inhibition. 3α,5α-Tetrahydroprogesterone (3α,5α-THP) and dexamethasone were less potent inhibitors. Patch-clamp techniques revealed that PROG co-applied with DMPP inhibited peak DMPP-induced current up to 68% and with 3 min pre-incubation inhibited both peak and integrated current up to ~95%. Monitoring of FURA-2 showed that PROG similarly inhibited parallel changes in intracellular-free $Ca^{++}$ concentration. PROG also inhibited CA secretion elicited by elevated $K^{+}$ (38%), and, in single cells, suppressed $Ca^{++}$ current evoked by step depolarization, inhibiting amplitude by 15%, and reducing the time constant of current decay during depolarization by 57%. In contrast to the immediate inhibition of nicotinic current, inhibition of $Ca^{++}$ current became statistically significant only after 1 min exposure to PROG. PROG did not inhibit secretion stimulated by high $Ca^{++}$ perfusion of permeabilized cells. These data suggest that PROG inhibits CA secretion from chromaffin cells predominantly by rapidly inhibiting nAChRs, and by gradually enhancing the inactivation of voltage-dependent $Ca^{++}$ channels (VDCCs), but not by affecting secretory processes downstream of $Ca^{++}$ influx. This study supports a role for adrenocortical PROG in the regulation of CA secretion during stress.


*Theme:* Endocrine and autonomic regulation
*Topic:* Neuroendocrine regulation: other

*Keywords:* Calcium ($Ca^{++}$); Catecholamines (CAs); Nicotinic acetylcholine receptors (nAChRs); Progesterone (PROG); Voltage-dependent calcium channels (VDCCs); Adrenal gland

## 1. Introduction

Progesterone (PROG) has long been known for its role in regulating reproductive function by binding to intracellular receptors and activating gene transcription [9,10]. Rapid, non-genomic actions of PROG on ion channels (ligand-gated, receptor- or voltage-operated) are now also recognized, and account for physiological phenomena as diverse as the initiation of the acrosome reaction in sperm [14], a decrease in contractility of vascular smooth muscle [47], and an inhibition of anxiety and epileptic seizures. The latter two effects likely occur via PROG conversion to its metabolite, 3α,5α-tetrahydroprogesterone (3α,5α-THP) in the CNS [36]. PROG has also been reported to rapidly inhibit CA secretion from chromaffin cells of the adrenal gland [19], potentially contributing to the acute regulation of the mammalian stress response [48]. The mechanisms behind PROG inhibition of CA secretion are unclear; however, chromaffin cells present several possible sites of action for PROG.

The secretion of CAs in chromaffin cells occurs by activation of nAChRs, subsequent opening of VDCCs, and the activation of exocytotic processes. PROG has been shown to non-competitively inhibit several subtypes of nAChRs including the $\alpha_4\beta_2$ neuronal [11,17,37,43], and the $\alpha_1\beta_1\gamma\delta$ human muscle and $\alpha_3\beta_4$ ganglionic nAChR [29]. While nAChR subunits $\alpha_3$, $\beta_4$, $\alpha_5$, and $\alpha_7$ are all expressed

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on bovine chromaffin cells [18,21], the $\alpha_3\beta_4$ ganglionic nAChR is believed to be predominantly responsible for CA secretion in these cells [24,42], presenting a potential target for the inhibition of CA secretion by PROG.

PROG may also inhibit CA secretion from chromaffin cells by affecting VDCCs. PROG inhibited KCl-induced increases in intracellular calcium concentrations in islet cells [41], and calcium current in smooth muscle cells [12,47], both of which were likely to occur by inhibition of the L-type VDCC. While still controversial, L-type VDCCs in chromaffin cells appear to contribute at least as much of the $Ca^{++}$ current controlling CA secretion [32] as either the N- or P/Q-type channels, which are also present on chromaffin cells [2,4,25]. Thus, the L-type VDCC is a possible site of action for PROG. PROG also moderately inhibited high $K^+$-induced CA secretion in chromaffin cells [19], suggesting inhibition of VDCCs. To date, however, there has been no direct evaluation of the effects of PROG on $Ca^{++}$ flux through VDCCs in chromaffin cells.

Finally, PROG may be inhibiting CA secretion in chromaffin cells by altering exocytotic processes downstream of $Ca^{++}$ influx, such as translocation, docking, priming, or fusion of chromaffin granules. High doses of PROG inhibited basal secretion of urokinase plasminogen activator from SKOV-3 human ovarian cancer cells by restricting the release of secretory vesicles [35], and these effects were non-transcriptionally mediated. Non-genomic effects of PROG on secretory processes downstream of $Ca^{++}$ influx in chromaffin cells, however, have yet to be examined.

The purpose of the present study was therefore to evaluate the acute, non-genomic effects of PROG on CA secretion in cultured bovine adrenal chromaffin cells, and to investigate the cellular mechanisms by which these effects occur.

## 2. Materials and methods

### 2.1. Materials

3α,5α-Tetrahydroprogesterone (3α,5α-THP) (5-α-pregnan-3-ol-20-one, 3α-hydroxy-5α-pregnan-20-one, allopregnanolone), and 4-pregnen-3,20-dion 3-*O*-carboxymethyloxime–bovine serum albumin (BSA) (BSA–PROG) were purchased from Steraloids, (Newport, RI), and FURA-2 AM was purchased from Molecular Probes (Eugene, OR). PROG, dexamethasone (DEX), DMPP, and all other chemicals were purchased from Sigma (St. Louis, MO).

### 2.2. Cell isolation and culture

Fresh bovine adrenal glands were obtained from a local slaughterhouse, and chromaffin cells were dissociated from adrenal medullae using digestion by collagenase (Sigma Blend C-8051 Type H). Glands were rinsed by injection into the renal vein of a divalent metal-ion-free Locke's solution (DMF-Locke's) consisting of the following (in mM): NaCl (145), HEPES (10), glucose (10), KCl (5.6), NaHCO3 (3.5), 100 units/ml penicillin, and 100 μg/ml streptomycin, with pH adjusted to 7.4. Glands were then injected with a 0.5% collagenase solution and shaken at 37 °C for 30 min. This step was repeated, after which medullary tissue was isolated from cortex and re-incubated in a beaker of fresh collagenase solution for another 30 min, 37 °C before filtration, pelleting and washing of cells, and resuspension in a 14% renograffin solution (Bracco Diagnostics). The suspension was then loaded under a 7.25% renograffin solution, centrifuged, and the interface collected and mixed with sterile Locke's solution (DMF Locke's plus 2.2 mM $CaCl_2$, and 1 mM $MgCl_2$). Cells were pelleted, washed again with Locke's, and resuspended in Dulbecco's Modified Eagle Medium (DMEM/F-12) supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml), gentamycin (10 μg/ml), fungizone (2.5 μg/ml), and cytosine β-D-arabinofuranoside (1 μM). For further purification, isolated cells were differentially plated by being suspended in media for 5 h in plastic flasks, with the non-adhered chromaffin cells then decanted and plated onto cell culture dishes (Becton Dickinson, France, USA) coated with calfskin collagen (0.1% in 0.1 M acetic acid). Cells were plated at a density of $1 \times 10^6$ cells/ml media and were maintained in primary culture in DMEM/F-12 (supplemented as above), until used for experiments 4–8 days after preparation. At least 48 h prior to any experiment media were replaced by media from which phenol red, serum, and fungizone were removed.

### 2.3. Determination of CA secretion

Cells were plated and maintained in 24-well plates (6.4 mm diameter wells) at $5 \times 10^5$ cells/0.5 ml media/well. For stock solutions, all steroids except BSA–PROG were dissolved in 100% EtOH. BSA–PROG was dissolved directly into physiological saline solution (PSS) that consisted of (in mM) NaCl (145), KCl (5.6), $CaCl_2$ (2.2), $MgCl_2$ (0.5), glucose (5.6), HEPES (15), and ascorbate (0.5) (pH 7.4). All test solutions were adjusted to contain 0.1% EtOH, the carrier concentration for the steroids, and at least 0.5% BSA. The 50-mM $K^+$ solution was identical to PSS except the $[K^+]$ was increased to 50 mM, and the $[Na^+]$ decreased to 100.6 mM. The DMPP solution was composed of DMPP dissolved in PSS to 20 μM, the concentration used in our lab to elicit maximal CA secretion in chromaffin cells. Using triplicate wells for each experiment, each experiment was repeated 2–6 times on different preparations of cells. During an experiment cells were maintained at 30 °C and pre-incubated for 10 min with PSS alone, before PSS was removed and test solutions added to each well for 2 min, unless otherwise specified. At the end of the 2-min stimulation period, test solutions that contained secreted CAs were removed and diluted in 5% trichloroacetic acid.

Cells remaining in the wells were then lysed with 5% trichloroacetic acid, and the lysate collected for determination of CA content remaining in the cells after stimulation. Samples were stored at 4 or −20 °C until assayed. CA content from the test solutions and the cell lysate was determined using a fluorometric assay [20] and percentage of total CA cell content secreted was calculated.

### 2.4. Electrophysiology

To determine the effects of PROG on the electrophysiological parameters of secretion, cells were trypsinized and re-plated onto glass cover slips, which had been attached by silicone adhesive to cover 4 mm round openings in 35 mm plastic dishes. Cells were re-plated onto cover slips coated with Cell Tak according to manufacturer's instructions, or with calfskin collagen. Cells on Cell Tak-coated slips were used for recording within 2–48 h of re-plating, and those on collagen-coated slips were used within 12–72 h of re-plating. Media were replaced with PSS approximately 30 min prior to an experiment, and all test solutions were adjusted to contain 0.1% EtOH, the carrier concentration for PROG.

Electrophysiological recordings were obtained by conventional whole-cell patch clamp techniques using an Axopatch 200 A amplifier (Axon Instruments, Foster City, CA) with Pulse Control software (Herrington and Bookman, University of Miami Medical School, Miami, FLA) run on an Apple G4 computer. Patch pipettes were pulled from borosilicate thin-walled glass tubes (1.5 mm o.d.) (A-M Systems, Inc, Carlsborg, WA) and fire-polished to a resistance of 2.5–7 MΩ.

Both $Ca^{++}$ and nAChR currents were filtered at 5 kHz, with $Ca^{++}$ current sampled at 25 kHz, and nAChR current at 6.25 kHz. For the recording of $Ca^{++}$ current, linear leak and capacitance current transients were subtracted using a P/4 protocol, and current recording was terminated when resting linear leak current was greater than 5% of the depolarization-induced current. Cells were maintained under general bath perfusion with solutions that consisted of either TEA ((in mM) tetraethylammonium (TEA)–Cl (130), $CaCl_2$ (10), HEPES (10), $MgCl_2$ (1), and glucose (10)) when recording $Ca^{++}$ current, or of PSS (see CA determination above) when recording nAChR current. Bath solutions were buffered with Tris to a pH of 7.3, maintained at a temperature of 21 °C, and then adjusted to within 5 mOsm of the given pipette-recording solution that ranged from 290 to 310 mOsm. For all experiments cells from which recordings were made were locally perfused within 50 μm by a glass pipette attached to solution reservoirs, with the rapid changing of solutions controlled by computer-automated valves. All cells were locally perfused with TEA until whole-cell configuration was achieved, and correct placement of the perfusion pipette was confirmed by switching local perfusion from TEA to PSS, and demonstrating that voltage-dependent $Na^+$ current could be evoked.

$Ca^{++}$ current was recorded in the TEA bath (±100 μM PROG) in response to step depolarizations to +10 mV made from a holding potential of −90 mV. In a subset of cells used for recording of $Ca^{++}$ current, the effects of PROG on $Na^+$ current were also tested. $Na^+$ current was recorded under local PSS perfusion ± 100 μM PROG, and evoked by step depolarizations to −20 mV from a holding potential of −90 mV. In experiments to obtain $Ca^{++}$ (and $Na^+$) current, the pipette solution containing (in mM) *N*-methyl-D-glutamine (NMDG)–Cl (140 mM), EGTA (10), HEPES (40), Mg-ATP (2), and GTP (0.3) was buffered to pH 7.1 with TRIS and adjusted to 290 mOsm. Nicotinic currents were recorded for 20 s intervals from a holding potential of −80 mV and were elicited by switching perfusion from PSS to PSS containing 20 μM DMPP (±PROG) for 10 s. Pipette solution used for recording nAChR current consisted of (in mM) KCl (140), HEPES (20), EGTA (10), MgATP (2), and GTP (0.3). To confirm complete amplitude of response to DMPP, cells were washed for 3 min between DMPP treatments, and at least two 10-s DMPP-elicited currents of equal amplitude were obtained before recording data. To minimize sensitization to PROG, each cell was exposed to only one concentration of PROG (1,10, or 100 μM). All data were analyzed using IGOR Pro software (Wavemetrics, Lake Oswego, OR, USA) on an Apple G4 computer.

### 2.5. Intracellular calcium imaging

To determine $\Delta[Ca^{++}]_i$, cells were trypsinized and re-plated onto glass cover slips in 35 mm plastic dishes, under identical conditions as described above for electrophysiological experiments. Media were replaced with PSS approximately 30 min prior to an experiment, and cells were subsequently loaded with the calcium indicator FURA-2 AM (1 μM) by bath perfusion for at least 20 min to allow for de-esterification. For enhanced cellular uptake FURA-2 AM was initially dissolved in dimethyl sulfoxide (DMSO) to 1 mM prior to dilution in PSS. All test solutions were adjusted to contain 0.1% EtOH, the carrier concentration for PROG, and cells from which recordings were made were locally perfused as described above.

To determine effects of PROG on DMPP-induced $\Delta[Ca^{++}]_i$, records were obtained by switching local perfusion for 10 s intervals to a solution of DMPP ± 100 μM PROG, or to DMPP + PROG after a 3-min pre-incubation with PROG. To control for repeated dosing with DMPP, two 10-s DMPP spikes of the same peak height were obtained on each cell before beginning data collection, and between treatments cells were washed with PSS for 2 min, the time determined to be necessary for full recovery of cells from a 10-s DMPP pulse.

Fluorescence of individual chromaffin cells was monitored by dual wavelength microspectrofluorometry (SPEX Industries, Edison, NJ) using a 40× oil immersion objective and alternating excitation wavelengths of 340 and 380 nm. Emission intensity was monitored at 510 nm and quantified

by a photon-counting photomultiplier. $[Ca^{++}]_i$ was calculated using the ratiometric method of Grynkiewicz et al. [23] where $[Ca^{++}]_i = (R - R_{min} / R_{max} - R)K_d \times F_o / F_s$. In this equation $R$ is the ratio of fluorescence at 340 nm excitation to that at 380 nm excitation, $K_d$ is the dissociation constant of FURA-2 (224 nM), $R_{min}$ and $R_{max}$ are the ratios for the $Ca^{++}$/FURA-2 complex when unbound (no $Ca^{++}$ and 10 mM EGTA), or bound (saturating $Ca^{++}$), respectively, and $F_o$ / $F_s$ = the ratio between minimal and maximal fluorescence of FURA-2 at 380 nm. For these experiments $R_{min}$ = 1.17, $R_{max}$ = 12.1, and Beta ($F_o$ / $F_s$) = 6.17.

### 2.6. CA secretion in permeabilized chromaffin cells

Cultured chromaffin cells were permeabilized and measurements of CA secretion were performed as previously described [26,46]. Chromaffin cells were pre-incubated in PSS after which the PSS was replaced for 4 min with a KGEP solution containing (in mM) potassium glutamate (139), PIPES (20) (pH 6.6), $MgCl_2$ (1), EGTA (5), Mg-ATP (2), Li-GTP (0.2), ascorbate (5), and 0.5% BSA that contained 20 μM digitonin to permeabilize cells. The digitonin-containing solution was then replaced for 2 min with treatment solutions of KGEP including 4.685 mM $CaCl_2$ buffered to 30 μM-free $Ca^{++}$ with EGTA, plus or minus varying concentrations of PROG (0.1–100 μM). Following the period of $Ca^{++}$-evoked release, extracellular KGEP solution was collected, and CA content in the test solution and cell lysate was determined as above.

### 2.7. Statistical analysis

Data are presented as means ± SEM. Statistical significance (set at $P < 0.05$) was determined by repeated measures ANOVA, or Student's $t$ tests (paired or unpaired). Bonferroni correction analysis was used when significance was determined from multiple comparisons. Statistical significance is indicated on data figures as: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

## 3. Results

### 3.1. Effects of PROG on CA secretion from intact cells

To confirm that PROG may exert direct and immediate regulatory effects on chromaffin cells we initially characterized the effects of acute PROG treatment on CA secretion from bovine chromaffin cells. While no significant effect was seen on basal secretion (data not shown), PROG dose-dependently (10–100 μM) inhibited CA secretion induced by 2 min exposure to the nAChR agonist, DMPP (20 μM), and at 100 μM inhibited CA secretion elicited by 2 min of exposure to 50 mM $K^+$. Maximal inhibition by PROG was 77 ± 1% for DMPP-induced secretion and 38 ± 3% for 50 mM $K^+$-induced secretion (Fig. 1A), suggesting that beyond

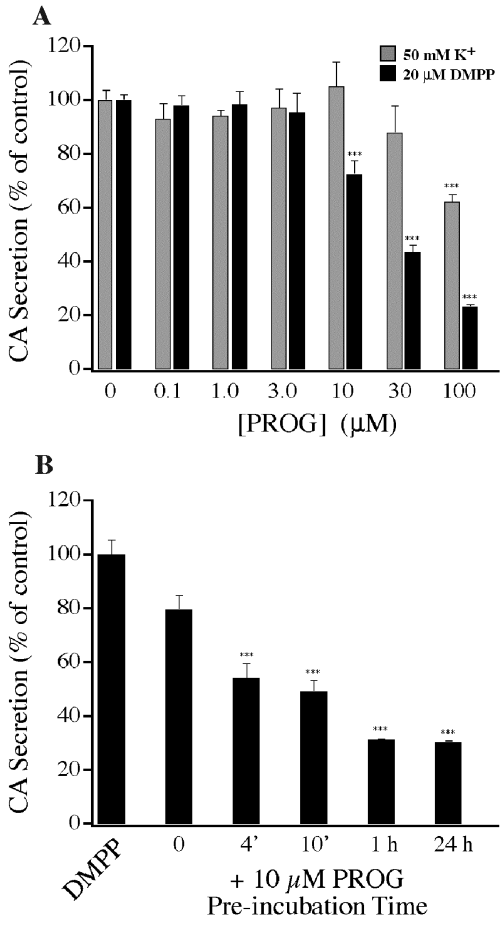


Fig. 1. Progesterone (PROG) inhibition of evoked CA secretion. (A) PROG, when applied simultaneously with agonist, dose-dependently inhibited CA secretion induced by 2 min stimulation with 50 mM $K^+$ (gray) or 20 μM DMPP (black). (B) Pre-incubation with 10 μM PROG prior to 2 min stimulation with DMPP and PROG enhanced inhibition by PROG. Data were normalized to percentage of control that included the PROG carrier EtOH equivalent to 0.1% and are expressed as means ± SEM. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

its effect on nAChRs PROG also inhibited VDCCs. When comparing the effects of PROG on CA secretion elicited by these two agonists, however, the differing degree of depolarization evoked by each agonist should be considered. For example, the actual values of CA release for these assays illustrate that 20 μM DMPP over 2 min stimulated an average release of 11.4 ± 0.4% of the total cell CA content ($n$ = 12), which was maximally inhibited by PROG to 2.6 ± 0.2%, while 2 min treatment of 50 mM $K^+$ treatment stimulated an average release of 6.2 ± 0.6% of total cell CA content ($n$ = 6), with maximal inhibition by PROG to 3.84 ± 0.08%.

Because the inhibition of DMPP-induced secretion was significant at 10 μM PROG, a dose more likely to be physiologically relevant than the 100 μM PROG necessary for inhibition of 50 mM $K^+$-induced secretion, we used DMPP to further characterize the effects of PROG on CA secretion. As chromaffin cells receive continuous low-level PROG exposure from adrenal cortical tissue in vivo [6,15],

which would increase with, for example, stress, we next tested whether pre-incubation with a sub-maximal dose of PROG would have a greater inhibitory effect on CA secretion than an immediate application. When cells were pre-incubated with 10 μM PROG prior to the 2-min co-application of DMPP + PROG the inhibition seen with 10 μM PROG increased from 21 ± 5% (no pre-incubation) to 46 ± 5%, 51 ± 4%, and 69 ± 0.6% with 4 min, 10 min, and 1 h, respectively, of pre-treatment with steroid (Fig. 1B). Inhibition was not further increased between 1 h and 24 h of pre-incubation with PROG. These data confirm the existence of an acute inhibitory effect of PROG on CA secretion in chromaffin cells that is markedly augmented with time of exposure up to 1 h.

At 30 and 100 μM, BSA–PROG, which cannot pass through the cell membrane, inhibited DMPP-induced secretion by 67 ± 2% and 94 ± 8% (Fig. 2A), which was slightly but not significantly more effective than unconjugated PROG at 30 and 100 μM. These data are consistent with inhibition of CA secretion occurring via a membrane-mediated mechanism. That CA inhibition by PROG does not involve an intracellular progesterone receptor is also supported by the fact that cells were maintained in serum-free, thus estrogen-free, media for 48 h prior to experiments and therefore lacked stimulus for expression of the nuclear progesterone receptor. The rapidity of PROG effects on secretion (2 min) also supports a non-genomic mechanism.

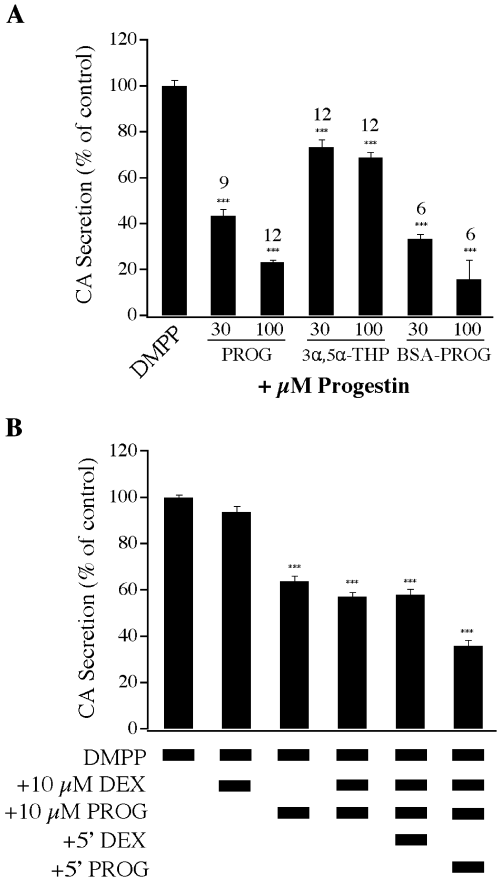


Fig. 2. Inhibition of evoked CA secretion by steroid analogs of PROG. (A) The PROG metabolite, 3α,5α-tetrahydroprogesterone (3α,5α-THP), was less effective at inhibiting DMPP-induced secretion (20 μM, 2 min) than PROG. PROG conjugated to BSA (BSA–PROG) inhibited DMPP-induced secretion similar to unconjugated PROG. (B) Dexamethasone (DEX) (10 μM) was a less effective inhibitor of DMPP-induced secretion than PROG (10 μM), when applied simultaneously or as a pre-treatment. Data were normalized to % of control that included the PROG carrier EtOH equivalent to 0.1% and are expressed as means ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

3α,5α-THP, a metabolite of PROG produced in the CNS that potently enhances $GABA_A$ receptor function [34], is reported to be responsible for the anxiolytic/protective effects of PROG [13], such as would occur during stress when CNS production of PROG is increased [38]. 3α,5α-THP is also produced in the adrenal gland [27] and increases in plasma following stress [22,38]. Consequently, we tested 3α,5α-THP to determine its ability to reduce CA secretion relative to that of PROG. With 3α,5α-THP at 30 and 100 μM, CA secretion induced by DMPP in chromaffin cells was reduced by 27 ± 3% and 31 ± 2% whereas inhibition by PROG was 57 ± 3% and 77 ± 1%, respectively (Fig. 2A). Moreover, the determination of full dose–response effects revealed that inhibition by 3α,5α-THP of DMPP-induced secretion was not significant at any concentration tested under 30 μM (0.1–10 μM), confirming that PROG itself was more effective than its metabolite, 3α,5α-THP, in the inhibition of DMPP-induced CA secretion.

Dexamethasone (DEX), a synthetic glucocorticoid, has been used previously to test the rapid, non-genomic effects of glucocorticoids on CA secretion in chromaffin cells [42]. Similar to the effects of PROG in the current study, DEX dose-dependently inhibited CA secretion (0–100 μM), although appeared slightly less potent than PROG. DEX achieved a maximal effect, at 100 μM, of 66% inhibition of CA secretion, while here PROG at 100 μM inhibited CA secretion by 77%. Thus, to begin to compare and distinguish the role of PROG from that of glucocorticoids, we compared the effects of a sub-maximal dose (10 μM) of PROG and of DEX on CA secretion (Fig. 2B). Without pre-incubation, 10 μM DEX inhibited DMPP-induced CA secretion by 6 ± 2% compared to 36 ± 3% by 10 μM PROG. To explore possible synergism between the two, DEX and PROG were then simultaneously applied to cells, and inhibition increased slightly to 43 ± 2%. Pre-treating cells with DEX for 5 min had no additional effect on the inhibition, yet pre-treatment with PROG for 5 min enhanced the inhibition to 65 ± 2%. Thus, at similar sub-maximal concentrations PROG exerted a stronger immediate inhibitory effect on CA secretion than DEX and exhibited an ability to increase inhibition with pre-incubation, while DEX had no such capability here.

### 3.2. Effects of PROG on DMPP-induced nAChR current

For a more direct measure of PROG effects on nAChR function, we determined the effect of PROG on stimulated

nAChR current in cells patch-clamped in the whole-cell configuration. Nicotinic current amplitude elicited by 10 s perfusion with 20 μM DMPP ranged from −126 to −1210 pA, with an average amplitude of −421 ± 106 pA ($n$ = 9). Effects of PROG were determined on both current amplitude ($I_{peak}$) and integrated area within each peak ($I_{integrated}$). When PROG was applied simultaneously for 10 s with DMPP, the $I_{peak}$ elicited by DMPP was significantly inhibited by 27% with PROG at 10 μM, and by 68% with PROG at 100 μM (Figs. 3A and B). The $I_{integrated}$ elicited by 10 s perfusion of DMPP was not significantly inhibited with 1 or 10 μM PROG, yet was inhibited by 73% ($P < 0.01$) with 100 μM PROG. PROG at 1 μM applied simultaneously with DMPP had no effect on either $I_{peak}$ or $I_{integrated}$ elicited by 10 s perfusion of DMPP (Figs. 3A and B).

When cells were pre-exposed to PROG for 3 min prior to the 10-s stimulation with DMPP, the inhibition of nicotinic current was markedly increased at all three concentrations of PROG tested (Figs. 3A and C). With pre-incubation, the DMPP-induced $I_{peak}$ was inhibited at 10 μM PROG by

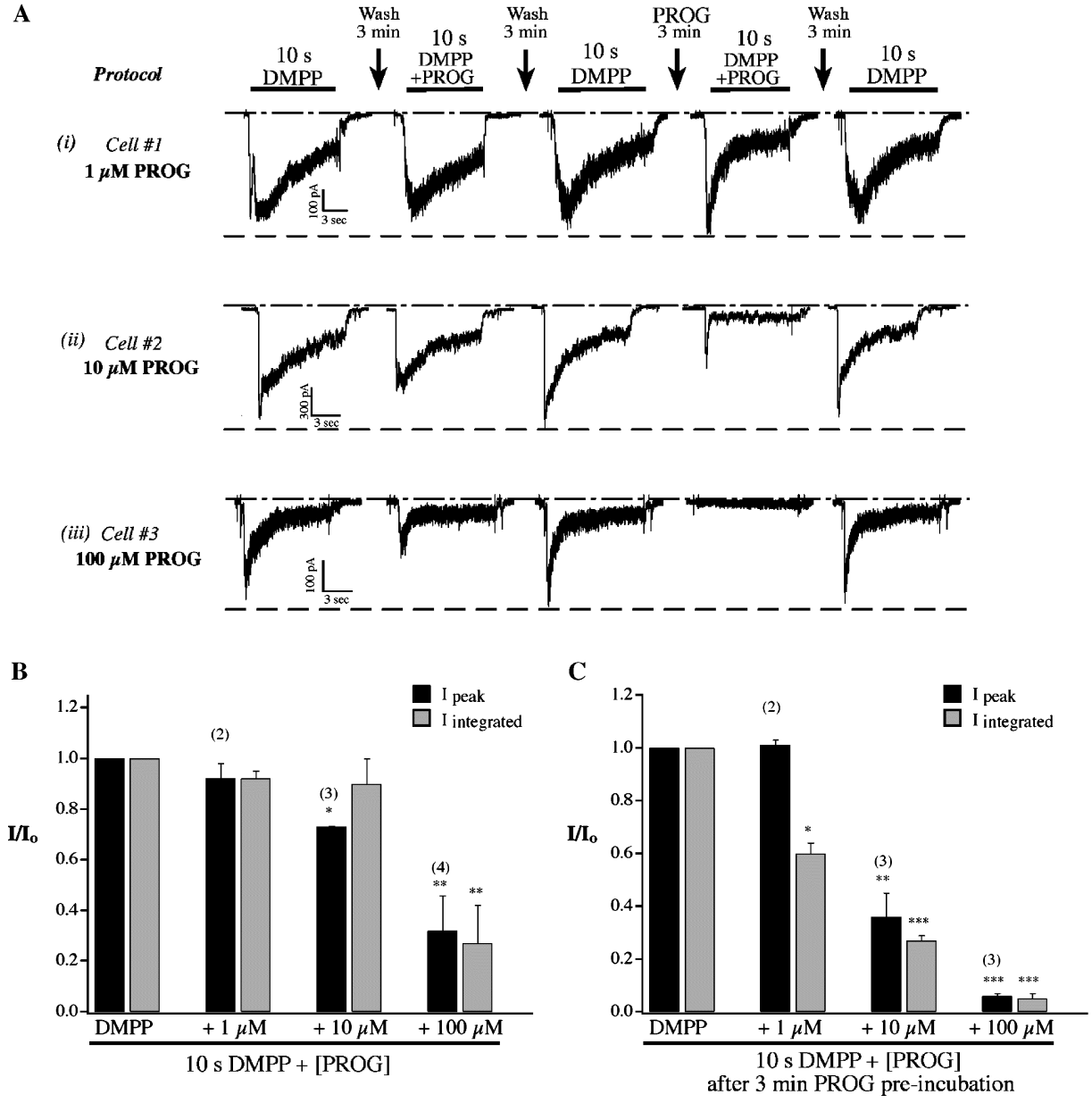


Fig. 3. PROG inhibition of DMPP-induced nicotinic ACh receptor (nAChR) current. (A) Recordings from 3 cells representing the effects of treatment by PROG (1 μM (i), 10 μM (ii), 100 μM (iii)) on nAChR current induced by 10 s pulse of 20 μM DMPP. Records of current show sequential responses obtained from single cells under voltage clamp in the whole-cell patch clamp configuration. Treatment conditions are indicated above and to the left of records (note time lapse indicated above current records). (B and C) Average inhibition of 10 s DMPP-induced current (current amplitude ($I_{peak}$—black) and integrated current ($I_{integrated}$—gray)) by PROG simultaneously applied with DMPP (B) or by PROG applied for 3 min prior to and during DMPP application (C). Number of cells is indicated over black bars. Inhibition is expressed as a fraction of initial DMPP-evoked current. Paired $t$ tests were used to determine statistical significance. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

64%, and at 100 μM PROG by 95% (Figs. 3A and C). In fact, two of the three cells tested at 100 μM PROG exhibited no visible peak current at all with the application of DMPP (i.e., Fig. 3A). $I_{integrated}$ then, elicited by this same protocol, was inhibited at 10 μM PROG by 73% and at 100 μM PROG by 95% (Figs. 3A and B). Importantly, 10 s perfusion of DMPP + 1 μM PROG after a 3-min pre-incubation with 1 μM PROG did not inhibit $I_{peak}$ of current elicited by 10 s DMPP alone, yet inhibited the $I_{integrated}$ by 40%, suggesting that PROG may have a compound effect on the nAChR.

### 3.3. Actions of PROG on stimulated $\Delta[Ca^{++}]_i$

To determine whether the inhibition by PROG on secretion in intact cells may be reflected also in altered $Ca^{++}$ signaling we monitored the fluorescence of single chromaffin cells that had been loaded with the $Ca^{++}$ indicator FURA-2 AM. The influence of PROG on $\Delta[Ca^{++}]_i$ induced by stimulation of the nAChR was assessed by local application of brief pulses (10 s) of DMPP in the presence or absence of PROG with at least a 2-min recovery given between pulses. PROG was found to inhibit the peak amplitude of $\Delta[Ca^{++}]_i$ induced by DMPP in control cells by 26 ± 10%, and a 3-min pre-incubation with steroid increased the inhibition to 59 ± 12% of control (Figs. 4A and B), confirming, again, enhanced inhibition with PROG pre-incubation, as seen in the nAChR current data, and the secretory dynamics.

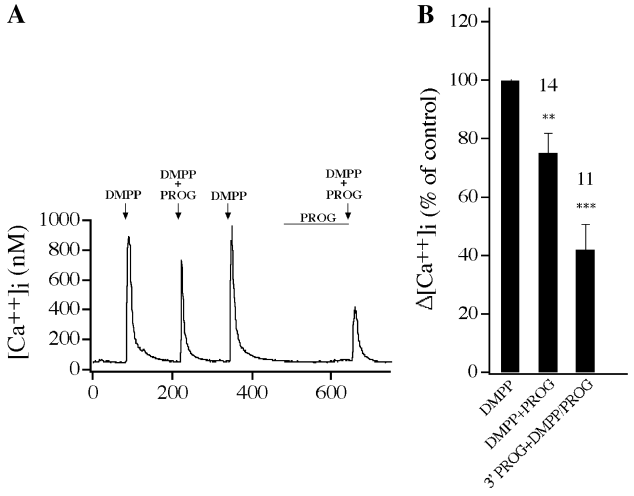


Fig. 4. PROG inhibition of DMPP-induced changes in intracellular calcium ($\Delta[Ca^{++}]_i$) in single cells loaded with FURA-2. (A) Representative example of $\Delta[Ca^{++}]_i$ elicited by 10 s pulses of DMPP (20 μM), and its inhibition by PROG (100 μM) applied either simultaneously or 3 min prior to DMPP application. Times of agonist and PROG application are indicated by bars above records. (B) Mean effects of 100 μM PROG on amplitude of $\Delta[Ca^{++}]_i$ elicited by 10 s exposure to 20 μM DMPP with or without pre-incubation by PROG. Data were normalized for each treatment as the % of $\Delta[Ca^{++}]_i$ relative to that of 10 s of DMPP alone. Number of cells tested is indicated over bars. Statistical significance was determined by paired *t* tests between $\Delta[Ca^{++}]_i$ in single cells. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

### 3.4. Effects of PROG on $Ca^{++}$ current evoked by step depolarization

Because PROG at 100 μM inhibited high $K^+$-induced CA secretion, it seemed possible that an effect of PROG on VDCCs may contribute to its inhibition of CA secretion elicited by stimulation of the nAChR. Thus, we investigated the effects of PROG on VDCCs, also using cells patch-clamped in the whole-cell configuration. Step depolarizations to +10 mV from a holding potential of −90 mV for 200 ms evoked $Ca^{++}$ current which averaged −319.8 ± 38 pA in amplitude ($I_{peak}$), and had an average time constant of current decay (tau) during the step depolarization of 0.585 ± 0.09 s ($n = 8$). $Ca^{++}$ currents evoked after 2 min perfusion with PROG (100 μM) showed significant inhibition of $I_{peak}$, integrated current ($I_{integrated}$), and tau (Figs. 5A and B). PROG treatment inhibited $I_{peak}$ $Ca^{++}$ current by 16 ± 5% ($P < 0.05$). More striking, however, PROG inhibited the $I_{integrated}$ by 32 ± 6% ($P < 0.001$) and enhanced the rate of decay of current, as expressed by a decrease in tau of 57 ± 6% ($P < 0.001$), thus suggesting that the major effect of PROG on VDCCs was to enhance the inactivation of the channels. These effects were specific to PROG as a 2-min perfusion with 100 μM cholesterol caused no significant inhibition (~2%) of any measure of $Ca^{++}$ current ($n = 4$) (Fig. 5B).

To examine the time course of PROG effects on VDCCs, a current was obtained prior to and immediately (1 s) following PROG application, and then subsequently in 30 s intervals up to 2 min upon which PROG was removed and recovery of the $Ca^{++}$ current followed (Figs. 6A and B). To reflect the most prominent effect of PROG on $Ca^{++}$ current, which was its enhancement of current decay, the time course of the effect of PROG was determined by comparing current amplitude at the end (195th ms) of the 200-ms step depolarization (Fig. 6A), imposed after varying durations of cell exposure to PROG (Figs. 6A and B). This comparison revealed that treatment with PROG for 1 and 30 s caused no significant inhibition of current. The first significant inhibition of evoked $Ca^{++}$ current (29%) was seen only after 60 s PROG, with inhibition increasing to 38% after 90 s, and 47% after 2 min of PROG exposure. Recovery from PROG inhibition occurred much more rapidly. After only 1 s of wash, current had recovered to 73% of its original value, and after 30 s of wash, 90% of the original current had been recovered (Fig. 6B).

### 3.5. Effects of PROG on CA secretion from permeabilized cells

Finally, to determine whether PROG exerted effects on CA secretion downstream of $Ca^{++}$ entry into the cell, we used cells permeabilized with digitonin, which allowed for direct access of $Ca^{++}$ to the intracellular space, and control of $[Ca^{++}]_i$ by buffering with EGTA. After permeabilization, cells were exposed for 3 min to a solution containing 30

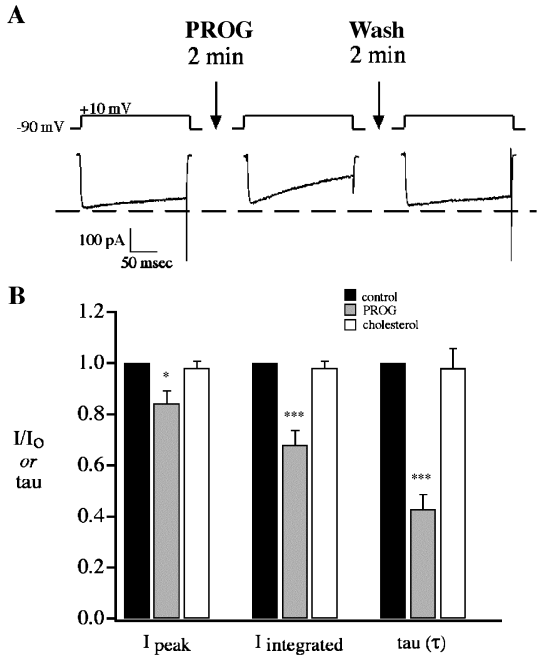


Fig. 5. PROG inhibits voltage-dependent $Ca^{++}$ current in chromaffin cells. (A) Representative example of the effect of PROG on $Ca^{++}$ current evoked by step depolarization to +10 mV from a holding potential of −90 mV. Record shows sequential currents taken from a single cell patch-clamped in the whole-cell configuration. (B) Bar graph summarizing the percent inhibition of $Ca^{++}$ current, reflected in the inhibition of current amplitude, integrated current, and rate of current decay (tau) (τ) after 2 min perfusion with 100 μM PROG (gray) or 100 μM cholesterol (white) ($n$ = 8).

μM-free $Ca^{++}$ to stimulate secretion, or 30 μM-free $Ca^{++}$ plus increasing concentrations of PROG (0.1–100 μM). The 30 μM-free $Ca^{++}$ stimulated cells to release on the average, 13.7 ± 0.4% of the total CA cell content ($n$ = 9). The addition of PROG at nearly all concentrations resulted in a limited but statistically significant enhancement of $Ca^{++}$-dependent secretion, with the largest augmentation seen with 1 and 10 μM of PROG (~16%) ($P < 0.001$) (Fig. 7). These data indicate that when $[Ca^{++}]_i$ is maintained constant, PROG does not inhibit CA secretion, suggesting that the inhibition by PROG of stimulated CA secretion in intact cells occurs by affecting mechanisms which alter $[Ca^{++}]_i$ concentrations.

## 4. Discussion

In this report we investigated the effects of PROG on CA secretion from chromaffin cells. At PROG levels which adrenomedullary chromaffin cells in vivo may be exposed to during stress, we have shown that PROG exerts multiple inhibitory effects on CA release. First, we provide direct evidence that PROG profoundly inhibits nAChR function in chromaffin cells, an effect that occurs immediately with agonist, and is augmented by pre-incubation with steroid. Second, we demonstrate that PROG inhibits VDCC function in chromaffin cells, that it appears to do so by enhancing the inactivation of these channels—a previously unrecognized mode of action by PROG on VDCCs, and that this effect is slightly delayed, requiring at least 1 min of PROG exposure. Third, we show that in chromaffin cells PROG does not inhibit secretion downstream of $Ca^{++}$ influx, suggesting that acute PROG treatment does not affect intracellular exocytotic machinery stimulated by $Ca^{++}$. Overall, our data provide a mechanistic explanation for the inhibition by PROG of CA secretion from chromaffin cells and suggest a role for PROG in the regulation of adrenomedullary CA secretion during stress.

At concentrations that may be physiologically relevant in the adrenal gland during stress, we have shown that PROG inhibited CA secretion induced by stimulation of the nAChR in a dose-dependent and time-dependent fashion. Resting (non-stress) adrenal vein PROG levels measure

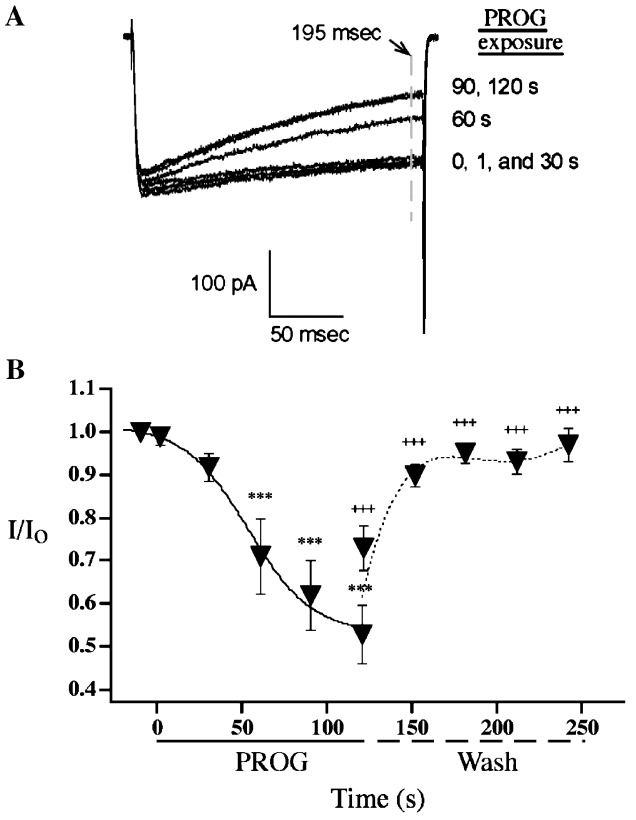


Fig. 6. Time course of PROG effects on voltage-dependent $Ca^{++}$ current. (A) Representative family of superimposed currents evoked by sequential 200 ms step depolarizations in a single cell taken every 30 s of PROG treatment. Dashed vertical line indicates the 195th ms of the 200-ms step depolarization (the time point at which amplitude of current was measured to determine the effect of PROG over time). (B) Time course of the inhibition by PROG (*) and of the recovery with wash (+) of current amplitude at the end (195th ms) of the 200-ms step depolarization. Data represent summary of sequential step depolarizations taken every 30 s of treatment on individual cells ($n$ = 8). Effects of PROG significantly different from control ($I_o$) (***$P < 0.001$) and effects of wash significantly different from 2 min PROG ($^{+++}P < 0.001$) were determined on raw data using repeated measures ANOVA with Bonferroni correction. Data were then standardized and shown here as means ± SEM.

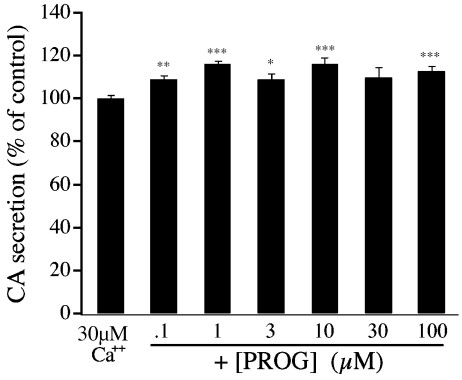


Fig. 7. PROG enhancement of CA secretion in permeabilized cells. CA secretion elicited by exposure of digitonin-permeabilized cells to 30 μM $Ca^{++}$ for 3 min was enhanced by co-exposure to PROG in a dose-independent manner. Results were normalized to secretion elicited by control + EtOH carrier (0.1%) and data expressed as means ± SEM ($n = 9$), with significance determined using Student's $t$ test with Bonferroni correction. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

~1.1 μM in cattle [6], and between ~1.3 to 2.3 μM in rat [15]. With peripheral serum PROG levels in cattle increasing from 10- to 22-fold by 15–30 min after ACTH injection [5,45] due solely to adrenocortical PROG production, it is possible that bovine chromaffin cells may be bathed with considerable concentrations of PROG (>10 μM) during stress. Consequently, while the normal fluctuations of PROG across a women's reproductive life cycle would not alter CA release by the mechanisms examined in these experiments, at PROG levels that are potentially present in the adrenal cortex during stress, we have shown that PROG dose-dependently inhibited CA secretion elicited by the nicotinic agonist, DMPP. Further, with increasing time of exposure, inhibition by 10 μM PROG increased up to 3.5-fold over the course of an hour, suggesting that concentrations of PROG less than 10 μM may also significantly inhibit CA secretion when cells are exposed for a time period longer than 2 min.

Mechanistically, the most striking and unquestionable result from these experiments is that the nAChRs in chromaffin cells are distinctly influenced by PROG. The inhibition of nAChR function by PROG was demonstrated consistently by all three methods used to test PROG effects on chromaffin cells. When the nAChR was stimulated by DMPP, PROG inhibited nAChR current, $\Delta[Ca^{++}]_i$, and CA secretion, and an enhanced inhibition of the nAChR by increased exposure to PROG was documented in each of these three parameters. Furthermore, the inhibition of DMPP-induced CA secretion by PROG was membrane mediated and more pronounced than inhibition caused by either the synthetic glucocorticoid, DEX, or the PROG metabolite, 3α,5α-THP.

This inhibition by PROG of nAChR function is most likely the result of action on the $\alpha_3\beta_4$ ganglionic nAChR, the nAChR believed to be predominantly responsible for CA secretion in bovine chromaffin cells [24,42]. The $\alpha_7$ homomeric nAChR is present on chromaffin cells [21] and has been reported to contribute to CA secretion under certain conditions [33], yet the majority of the literature has shown this receptor to have no effect on secretion [1,16,42]. Consequently, a 77% inhibition of secretion by PROG implies action on the $\alpha_3\beta_4$ receptor, though does not eliminate a possible effect on the $\alpha_7$ receptor as well.

The effects of PROG on the nAChR in chromaffin cells that we have demonstrated here using a multi-disciplinary approach are consistent with PROG inhibition of $\alpha_3\beta_4$ nAChRs in a cell line [29], and of ACh-induced CA secretion in chromaffin cells [19]. Additionally, our data demonstrate that enhanced inhibition of the nAChR by pre-incubation with PROG is consistent with pharmacological theoretical models of steroid inhibition of nAChRs. A single high affinity binding site for allosteric inhibitors on the extracellular portion of the nAChR channel has been proposed, as have several low affinity binding sites, possibly at the lipid–protein interface [3]. The immediate inhibition by PROG when co-applied with DMPP could be explained by initial contact of PROG with the proposed extracellular binding site, while its augmented inhibition with pre-incubation may be due to the eventual accumulation of steroid in the plasma membrane to access low affinity binding sites.

In this study PROG also inhibited VDCC function in chromaffin cells, demonstrated directly by the inhibition of evoked $Ca^{++}$ current in single cells, and indirectly by the inhibition of CA secretion elicited by a high $K^+$ (thus depolarizing) solution. The manner in which PROG inhibited $Ca^{++}$ current in chromaffin cells provides interesting and novel information on the effects of PROG on VDCCs. While PROG moderately inhibited current amplitude, the largest effect of PROG on $Ca^{++}$ current was the enhancement of the rate of current decay during depolarization, suggesting that the most prominent effect of PROG was to enhance the inactivation of a population of VDCCs contributing to this current. Second, with repeated step depolarizations, the inhibition of $Ca^{++}$ current increased to statistically significant levels only after 1 min of exposure and continued to increase up to the longest time interval studied (2 min). Because N-, P/Q-, and L-type channels all contribute to high-voltage-activated $Ca^{++}$ current in bovine chromaffin cells [2,4,25], PROG could theoretically be acting on one or more of these channel types. While PROG inhibition of $Ca^{++}$ current through L-type channels has been reported in vascular cells [7,12,47] this inhibition was consistently achieved by inhibition of the amplitude of the current, and not by an effect on the rate of current decay. Our data then suggest that in the chromaffin cell, PROG either exerts a novel type of inhibition on the L-type channel, which is of a different subunit composition than those in vascular cells, or is exerting effects on additional VDCC types. Either action is interesting not only in the context of chromaffin cell and stress physiology, but also in examining the role of PROG as a neuroactive steroid. In the

brain, PROG is present in high quantities [8], increases during stress [38], and is associated with protective effects such as decreasing anxiety [13], and decreasing susceptibility to epileptic seizures [39]. Thus, information contributing to our understanding of PROG effects on VDCCs, several of which are present in the brain, may have implications for systems beyond the chromaffin cell.

This study suggests that PROG, as a potential stress hormone, appears to play a distinctive role as one member of a growing family of adrenocortical steroids that inhibit CA secretion from chromaffin cells. PROG was a more potent inhibitor of CA secretion than DEX or 3α,5α-THP, and a previous report showed PROG to inhibit CA secretion in chromaffin cells to a greater extent than several other steroids tested [19]. Here, PROG did not inhibit CA secretion stimulated by excess $Ca^{++}$ in permeabilized cells, suggesting that PROG does not affect exocytotic processes downstream of $Ca^{++}$ entry into the cell. This contrasts with dehydroepiandrosterone (DHEA), an adrenocortical androgen inhibiting CA secretion that does appear to modulate intracellular secretory processes [31]. As well, PROG inhibited VDCCs in this study, whereas the glucocorticoid, DEX [28,44], and the androgens, DHEA [31], and DHEA sulfate [30], have been reported to inhibit CA secretion in chromaffin cells without directly affecting VDCCs. Of note, alphaxalone, a synthetic anesthetic progestin, also has demonstrated inhibitory effects on VDCCs in chromaffin cells [40], suggesting, possibly, that progestins as a class, including metabolites such as 3α,5α-THP, may inhibit VDCCs.

The specific role that PROG plays in the adrenocortical steroid system, and the actual physiological relevance during a stress response in vivo of adrenocortical PROG, remains to be defined. It seems likely that these steroids that inhibit CA secretion in vitro, and that are produced by the cortex and bathe chromaffin cells of the medulla during stress before entering the general circulation, may participate in a type of complex adrenal auto-regulation. This auto-regulatory system could protect an organism from excess exposure to CAs and thus cardiovascular morbidity. Further investigation, however, is required before the actual in vivo benefit of CA inhibition by PROG or any other adrenocortical steroid can be confirmed.

In summary, we have provided data demonstrating that, in chromaffin cells, PROG strongly and rapidly inhibits the nAChR, which in turn effects a potent inhibition of CA secretion. We have also demonstrated that in these cells PROG exerts a gradual inhibition of VDCCs, an action that has the potential to contribute to the inhibition of CA secretion under certain conditions, and may have implications for PROG action in the brain. PROG did not affect intracellular exocytotic machinery. This study, then, clarifies the effects of PROG on chromaffin cells, suggests that PROG as an adrenocortical steroid may play a distinct role in the inhibition of CA secretion during stress, and contributes to our understanding of PROG as a protective hormone.

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