# An extracellular sulfhydryl group modulates background Na + conductance and cytosolic Ca2+ in pituitary cells

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*Am J Physiol Cell Physiol* 282:864-872, 2002. First published Nov 21, 2001;
doi:10.1152/ajpcell.00441.2001

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# An extracellular sulfhydryl group modulates background $Na^+$ conductance and cytosolic $Ca^{2+}$ in pituitary cells

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Received 13 September 2001; accepted in final form 16 November 2001

**Fonteriz, Rosalba I., Carlos Villalobos, and Javier García-Sancho.** An extracellular sulfhydryl group modulates background $Na^+$ conductance and cytosolic $Ca^{2+}$ in pituitary cells. *Am J Physiol Cell Physiol* 282: C864–C872, 2002. First published November 21, 2001; 10.1152/ajpcell.00441.2001.—Treatment of $GH_3$ pituitary cells with *p*-chloromercurybenzenesulfonate (PCMBS) increased the cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_i$). This effect was reversed by dithiothreitol and blocked by L-type $Ca^{2+}$ channel antagonists or $Na^+$ removal. PCMBS increased membrane conductance and depolarized the plasma membrane. Apart from minor effects on $K^+$ and $Ca^{2+}$ channels, PCMBS increased (6 times at −80 mV) an inward $Na^+$ current whose properties were similar to those of a background $Na^+$ conductance (BNC) described previously, necessary for generation of spontaneous electrical activity. In rat lactotropes and somatotropes in primary culture, PCMBS also produced a $Na^+$-dependent $[Ca^{2+}]_i$ increase, whereas little or no effect was observed in thyrotropes, corticotropes, and gonadotropes. The $Na^+$ conductance elicited by PCMBS in somatotropes seemed to be the same as that stimulated by the hypothalamic growth hormone (GH)-releasing hormone, which regulates membrane excitability and GH secretion. The BNC studied here could play a physiological role, regulating excitability and spontaneous activity, and explains satisfactorily the $[Ca^{2+}]_i$-increasing actions of the mercurials reported previously in several excitable tissues.

$GH_3$ cells; sodium current; mercurials; *p*-chloromercurybenzenesulfonate; neurotoxicity

MERCURY ($Hg^{2+}$) is a common environment contaminant. Both organic and inorganic forms are neurotoxic, and the hypothesis that the effects of $Hg^{2+}$ can be mediated by changes in the intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) has received considerable attention (3, 8). For example, in PC-12 cells $Hg^{2+}$ is able to produce an increase in $[Ca^{2+}]_i$ due to $Ca^{2+}$ entry through voltage-gated $Ca^{2+}$ channels, which can lead either to cell differentiation or to cell death, depending on the $Hg^{2+}$ concentration used (31). Several effects on plasma membrane ion channels, mainly $K^+$ and $Ca^{2+}$ channels, have been reported, but they do not explain the observed changes in $[Ca^{2+}]_i$ (6, 13, 16, 20, 22, 25, 34). In addition, most of these studies have been carried out with membrane-permeant mercurials, which can act, with different time lags, at both the extracellular and the intracellular side, thus complicating the interpretation of the results.

Here we find that the membrane-impermeant mercurial *p*-chloromercurybenzenesulfonate (PCMBS) produces a large increase of $[Ca^{2+}]_i$ in pituitary cells. A systematic study of the actions of PCMBS on the different plasma membrane ionic currents reveals that, apart from minor effects on $K^+$ and $Ca^{2+}$ channels, the action on $[Ca^{2+}]_i$ arises from activation of an inward $Na^+$ current and membrane depolarization. The activity of this $Na^+$ conductance controls the firing rate in $GH_3$ pituitary cells and in lactotropes (32, 36). Growth hormone-releasing hormone (GHRH) is known to act on somatotropes by stimulating a similar $Na^+$ conductance (18, 19, 21, 29, 37). Thus, in addition to explaining the mechanism for the stimulation of $Ca^{2+}$ influx by mercurials, our results suggest that a sulfhydryl group facing toward the extracellular side of the membrane may be involved in regulation of the activity of pituitary cells. It has been shown recently that regulation of plasma membrane permeability to $Na^+$ may be crucial for control of membrane potential, firing rate, and secretion in other endocrine cells (23, 24).

## METHODS

$GH_3$ pituitary cells and rat anterior pituitary (AP) cells were prepared and grown as described previously (39, 40, 42). The cells were seeded over glass coverslips coated with poly-L-lysine (0.01 mg/ml, 10 min). Enriched somatotropes were prepared as follows: 1–3 × $10^6$ freshly prepared AP cells were suspended in 0.5 ml of standard medium (see composition below) containing 40% Percoll. This cell suspension was placed into an Eppendorf tube on top of a discontinuous Percoll gradient formed by the following layers (from top to bottom): 40% Percoll (0.2 ml), 50% Percoll (0.2 ml), and 80% Percoll (0.1 ml). After a 5-min centrifugation at 3,000 *g*, the enriched cell population (~10% of the starting cells) was recovered at the 50–80% interphase, washed with standard medium, and plated on coverslips as described above. Immunocytochemistry showed that 73 ± 2% of these cells stored growth hormone (mean ± SE; $n$ = 5).

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$[Ca^{2+}]_i$ measurements were performed by time-resolved digital image analysis in fura 2-loaded cells as described previously (39, 40, 42). The standard incubation medium had the following composition (in mM): 145 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 10 sodium-HEPES, pH 7.35. The high-$K^+$ (50 mM) solutions were prepared by replacing 45 mM of NaCl by KCl. All experiments were performed at 37°C. At the end of the $[Ca^{2+}]_i$ measurements, AP cells present in the microscopic field were typed by multiple sequential primary immunocytochemistry by using antibodies against the pituitary hormones labeled with Oregon green 488 (41, 43). This allowed identification of up to three different cell types (e.g., somatotropes, lactotropes, and corticotropes in Fig. 9) in the same microscope field. $Mn^{2+}$ entry was measured by quenching of fura 2 fluorescence as described previously (39, 40).

Patch-clamp experiments were performed on a Nikon Diaphot 200 inverted microscope by using an Axopatch-1D patch-clamp amplifier (Axon Instruments, Foster City, CA). For recording and analysis we used WCP software (John Dempster, Dept. of Physiology & Pharmacology, Strathclyde University, Glasgow, Scotland). Coverslips with cells were placed on a Lucite chamber (volume 200 μl) on the microscope stage and perfused at ~2 ml/min at room temperature (22–28°C). Bath exchanges were accomplished by switching the bath inflow line. Both standard whole cell patch-clamp (12) and patch-perforated (15) methods were employed for electrophysiological recording. Perforated patches were achieved by using nystatin (final concentration 500 μg/ml). Patch electrodes had tip resistances of 4–7 MΩ. Access resistance was determined from the series resistance compensation dial on the patch amplifier. In standard whole cell recordings, access resistances ranged between 5 and 55 MΩ. Perforated patches reached stable access resistances (20–40 MΩ) after 5–10 min. An Ag-AgCl pellet placed in the efflux line of the chamber was used as a ground electrode. The offset potential between the pipette and the bath (1–4 mV) was compensated with the patch-clamp amplifier. No series resistance compensation was used. Corrections of the voltage drop across the access resistance for the calculation of reversal potential were not made because the current was very small and the voltage artifact negligible.

The standard bath solution was as described above for $Ca^{2+}$ measurements. In the $Na^+$ substitution experiments, NaCl was replaced by equivalent amounts of *N*-methyl-D-glucamine (NMDG) or tris(hydroxymethyl)aminomethane (Tris), adjusted to neutral pH with HCl. For measurements of $Ca^{2+}$ currents, 10 mM $CaCl_2$ replaced an equivalent amount of NaCl. For measurements of $Na^+$ currents, the external solution contained (in mM) 150 NaCl, 1 $MgCl_2$, 10 glucose, 5 HEPES-Tris, pH 7.35, 2 $NiCl_2$ (to block $Ca^{2+}$ currents), 1 $BaCl_2$, and 2 CsCl (to block the delayed rectifier $K^+$ currents). Pipette solutions for standard whole cell recording contained (in mM) 140 KCl, 2 $MgCl_2$, 0.7 $CaCl_2$, 1.1 EGTA (free $Ca^{2+}$ concentration 100 nM), and 10 sodium-HEPES, pH 7.2. Pipette solutions for perforated-patch recording contained (in mM) 65 KCl, 30 $K_2SO_4$, 10 NaCl, 1 $MgCl_2$, 50 sucrose, and 20 potassium-HEPES, pH 7.2. Pipette solution for recording $Ca^{2+}$ currents and background $Na^+$ conductance contained (in mM) 65 CsCl, 30 $Cs_2SO_4$, 10 NaCl, 1 $MgCl_2$, 50 sucrose, and 20 cesium-HEPES, pH 7.2.

Antisera against pituitary hormones were the same batches cited previously (41, 43). Furnidipine was a generous gift from Laboratorios Alter (Madrid, Spain). Calciseptine was purchased from Latoxan (Rosans, France). Fura 2-AM and Oregon green 488-isothiocyanate were purchased from Molecular Probes (Eugene, OR). Other chemicals were obtained from either Sigma (Madrid, Spain) or Merck (Darmstadt, Germany).

## RESULTS

*Treatment with PCMBS increases $[Ca^{2+}]_i$ in $GH_3$ cells.* Figure 1*A* shows the effects of treatment with PCMBS (50 μM) in $GH_3$ cells loaded with fura 2. After a lag of ~30 s, $[Ca^{2+}]_i$ increased from the resting value of ~100 nM to 800 nM. Washing PCMBS did not

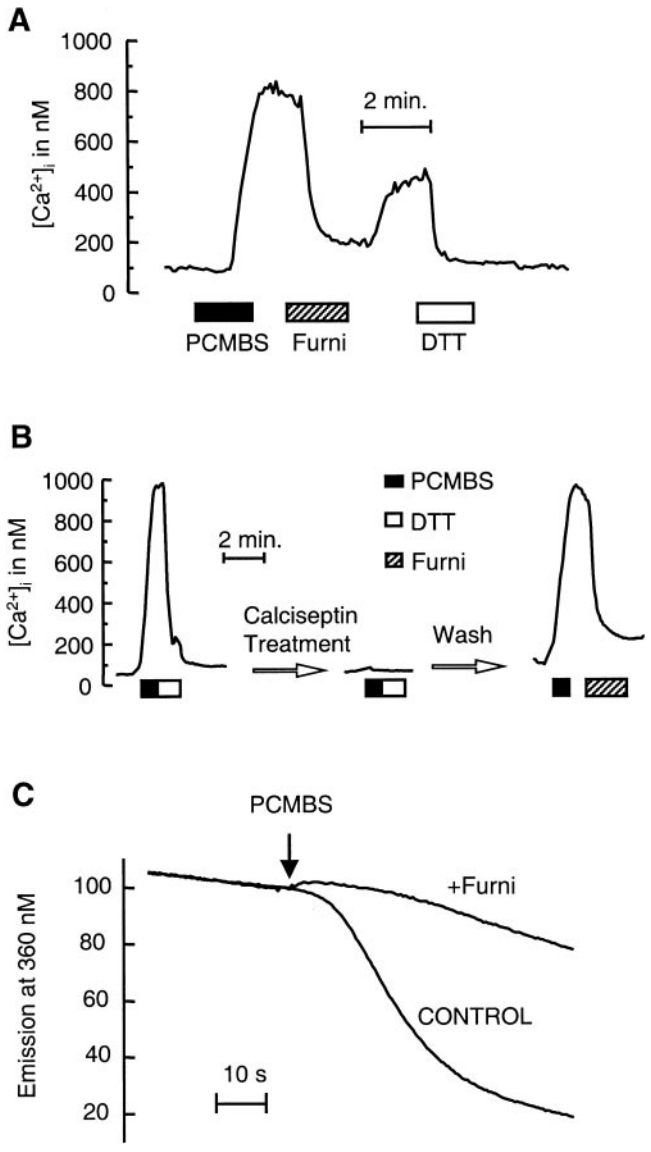


Fig. 1. Treatment with *p*-chloromercurybenzenesulfonate (PCMBS) increases $[Ca^{2+}]_i$ and accelerates $Mn^{2+}$ entry in $GH_3$ pituitary cells. Concentrations of PCMBS, furnidipine, and dithiothreitol (DTT) were 50, 1, and 2,000 μM, respectively. *A*: average of 44 cells present in the same microscope field. *B*: average of 112 cells; cells were treated with 1 μM calciseptine for 20 min and then washed with fresh medium for 15 min. *C*: $MnCl_2$ (0.5 mM) was added 30 s before PCMBS. Furnidipine was added 30 s before $Mn^{2+}$. Traces corresponding to control and furnidipine-treated cells are superimposed. Fluorescence emission is represented as a percentage of the value at the time PCMBS was added. The traces shown are representative of 3–5 similar experiments. $[Ca^{2+}]_i$, intracellular $Ca^{2+}$ concentration; Furni, furnidipine.

reverse the effect. Perfusion with furnidipine (1 μM), a dihydropyridine antagonist of L-type $Ca^{2+}$ channels (26), decreased $[Ca^{2+}]_i$ toward the resting values [85% decrease of the change ($\Delta[Ca^{2+}]_i$)]. When the dihydropyridine was washed, $[Ca^{2+}]_i$ rose again, indicating that the $[Ca^{2+}]_i$-increasing ability of PCMBS was still present. Perfusion with the sulfhydryl-reducing agent dithiothreitol (DTT; 2 mM) at this time restored $[Ca^{2+}]_i$ to resting levels. When DTT was washed, no further changes in $[Ca^{2+}]_i$ took place, indicating permanent reversion of the effects of the mercurial. Single-cell analysis showed that all the $GH_3$ cells were similarly responsive to PCMBS. Figure 1*B* shows the effects of the specific L-channel blocker calciseptine (9). After treatment with PCMBS and reversion of the $[Ca^{2+}]_i$ increase with DTT, the cells were incubated with calciseptine (1 μM) for 20 min. A new treatment with PCMBS at this time had no effect on $[Ca^{2+}]_i$. After a 15-min wash of calciseptine, a new treatment with PCMBS again produced the $[Ca^{2+}]_i$ increase, which was antagonized by furnidipine. Thus our results suggest that the increase of $[Ca^{2+}]_i$ induced by PCMBS is due to $Ca^{2+}$ entry through L-type $Ca^{2+}$ channels. The membrane-impermeant HS reagents (2-aminoethyl)-methanethiosulfonate and (2-sulfonatoethyl)methanethiosulfonate (introducing positive and negative net charge, respectively; Ref. 1) also induced an increase of $[Ca^{2+}]_i$, which was reversed by DTT (results not shown).

$Mn^{2+}$ is able to permeate through L-type $Ca^{2+}$ channels (38). In Fig. 1*C*, $Mn^{2+}$ was used as a tracer for $Ca^{2+}$ entry. The entry of $Mn^{2+}$ was estimated from the quenching of the fura 2 fluorescence excited at 360 nm, a wavelength that is insensitive to $[Ca^{2+}]$ (40). After a brief lag (~15 s), treatment with PCMBS increased the rate of fluorescence quenching 16-fold. In the presence of furnidipine, the effect of PCMBS was nearly abolished.

*Effects of PCMBS on membrane potential and excitability.* To identify the target of PCMBS, we studied its effects on the electrical activity of $GH_3$ cells. Figure 2 shows a typical experiment of membrane voltage measurement in the current-clamp mode. Treatment with PCMBS caused depolarization of the cell membrane. The lower traces in Fig. 2 show time-expanded records of spontaneous action potentials recorded under the control condition (*left*), after addition of PCMBS (*middle*), and after exposition to DTT (*right*). PCMBS depolarized the cells, decreased the amplitude of the action potentials, and, in most cases, increased the firing frequency. These effects were reversed by DTT. In 22 similar experiments, the mean depolarization (±SE), measured 3 min after treatment with PCMBS, was 15 ± 2 mV. The membrane depolarization and the increase of firing frequency could explain the increase of $[Ca^{2+}]_i$. We next investigated which membrane current could be the one responsible for the depolarizing effect of PCMBS.

*Effect of PCMBS on $Ca^{2+}$ and $K^+$ currents.* $Ca^{2+}$ currents were the first candidates to explain the observed effects. Whole cell $Ca^{2+}$ currents were recorded by using the perforated-patch technique with $Cs^+$-loaded micropipettes and elevated (10 mM) bath $Ca^{2+}$ (see METHODS for details). To study the effect on the noninactivating $Ca^{2+}$ currents, we held cells at −40 mV and then depolarized to +40 mV in 10-mV voltage jumps. Figure 3*A* shows the average of the current-voltage (*I-V*) curves obtained in four similar experiments. There was no significant difference between the mean values obtained in control and PCMBS-treated cells (Student's *t*-test, paired data). The peak $Ca^{2+}$ current was not significantly modified by PCMBS either. In another set of experiments cells were held at −80 mV and depolarized to +10 mV. In the average of three experiments there was no effect of PCMBS on transient currents (Fig. 3*B*), although in one case there was a small inhibition of the current (Fig. 3*C*). Thus the increase of $[Ca^{2+}]_i$ cannot be explained by an action of PCMBS on $Ca^{2+}$ channels.

Membrane depolarization by inhibition of $K^+$ channels would favor activation of voltage-gated $Ca^{2+}$ channels and $Ca^{2+}$ entry. We therefore studied the effects of

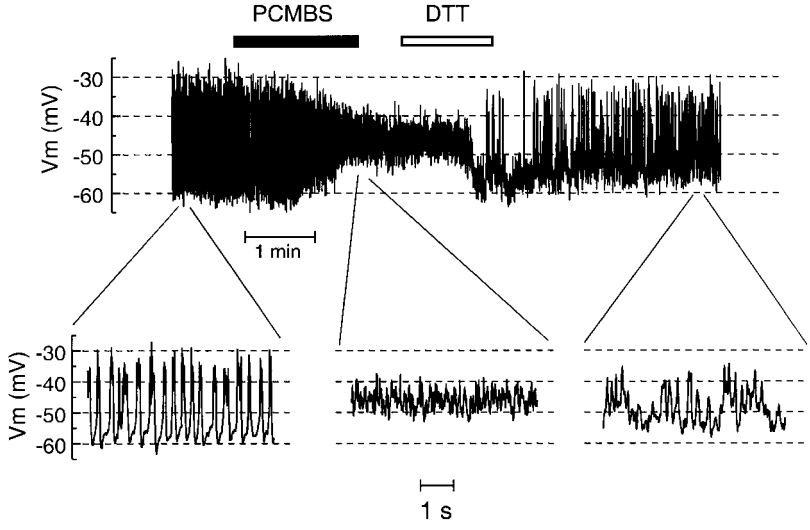


Fig. 2. Effects of PCMBS on membrane potential ($V_m$) and spontaneous firing of $GH_3$ cells. *Top*: perforated-patch measurements of electrical activity of $GH_3$ cells in current-clamp mode. *Bottom*: time-expanded records of spontaneous action potentials in the control condition (*left*), after addition of 50 μM PCMBS (*middle*), and 3 min after washing with the reducing agent DTT (2 mM) (*right*). Results are representative of 22 similar experiments.

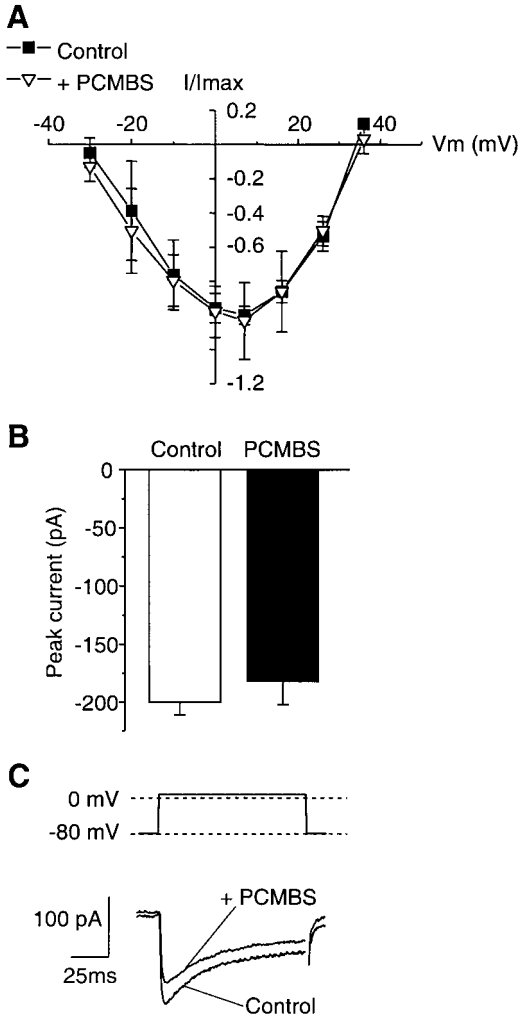


Fig. 3. *A*: current-voltage (*I-V*) curves for $Ca^{2+}$ currents elicited by 10-mV step depolarizations to 40 mV from a holding potential of −40 mV in the control condition and after PCMBS 50 μM treatment (+PCMBS), measured by perforated patch. Values are means ± SE of 4 experiments. *B*: bar graph representing average of peak currents elicited by depolarization to +10 mV from a holding potential of −80 mV in the control condition and after perfusion of PCMBS ($n$ = 3). *C*: example of $Ca^{2+}$ current record used to calculate data in *B*.

PCMBS on $K^+$ currents. Dubinsky and Oxford (10) described two different outward $K^+$ currents in $GH_3$ cells, one compatible with the delayed rectifier or voltage-dependent $K^+$ channel ($I_{Kv}$) and the other with $Ca^{2+}$-activated $K^+$ channels ($I_{KCa}$) (see also Ref. 30). The whole outward $K^+$ current was measured by applying 100-ms square depolarizing pulses in 10-mV steps from a holding potential of −50 mV. Figure 4*A* shows the normalized *I-V* plot constructed with the average results of seven experiments. There was a 30% inhibition at the more depolarized potentials, but the difference was not significant (Student's *t*-test, paired data). In another set of experiments, 0.2 mM $CdCl_2$ was added to the bath to block voltage-dependent $Ca^{2+}$ channels and, consequently, $Ca^{2+}$-dependent $K^+$ channels (Fig. 4*B*). Under these conditions, no differences were observed in the outward currents recorded before and after PCMBS treatment, suggesting that $I_{Kv}$ is not affected by PCMBS.

To explore directly the effect of the mercurial on $I_{KCa}$, we used a double pulse protocol (35) (Fig. 4*C*). Cells were first depolarized to +75 mV for 0.5 s from a holding potential of −85 mV. During this pulse, voltage-dependent $K^+$ channels are activated but $I_{KCa}$ are not, because very little $Ca^{2+}$ enters the cell at this positive potential. The membrane potential was then returned to 0 mV for 100 ms to allow $Ca^{2+}$ to enter the cell, and a second depolarizing pulse to +75 mV was applied for 2 s. This results in a second outward $K^+$ current carried mainly through $Ca^{2+}$-dependent $K^+$ channels (35). The transient current elicited by the first pulse was not modified by the mercurial. The outward current during the second pulse was partially inhibited by PCMBS in four of eight cells studied and was increased in the other four cells. The average of all the values was an inhibition of 13 ± 9% (mean ± SE). This small effect of PCMBS on $K^+$ currents does not seem large enough to explain the increase of $[Ca^{2+}]_i$.

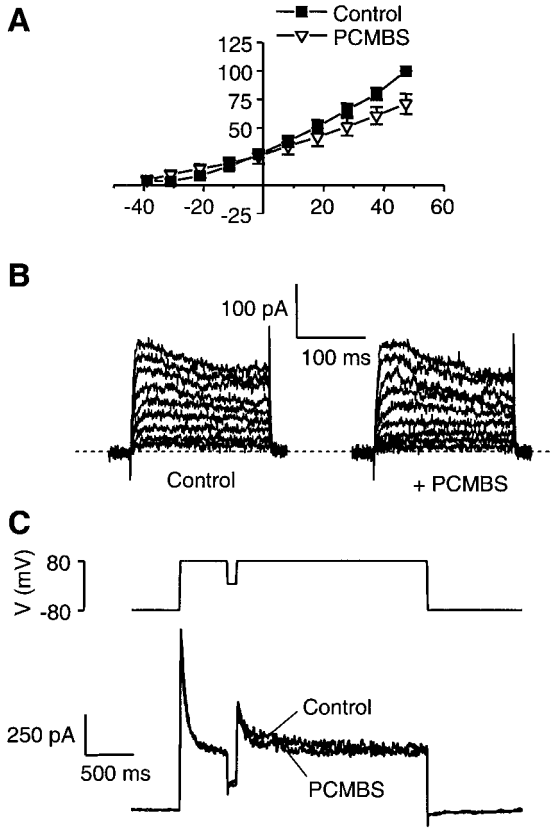


Fig. 4. Effects of PCMBS on $K^+$ currents. *A*: *I-V* representation of averaged whole cell recordings of outward $K^+$ currents elicited by 10-mV step depolarizations during 100 ms, from a holding potential of −50 mV in the control condition and after PCMBS treatment ($n$ = 7). *B*: $K^+$ currents recorded in the presence of 0.2 mM $CdCl_2$ from a holding potential of −50 mV, elicited by 10-mV step depolarizations to +40 mV. *C*: $K^+$ currents recorded with the double pulse protocol. Cells were depolarized to +75 mV from a holding potential of −85 mV for 500 ms, then repolarized to 0 mV for 100 ms, and finally depolarized again to +75 mV for 2 s. Results are from 1 of 8 similar experiments.

This conclusion is further supported by the observation that treatment of the cells with inhibitors of $Ca^{2+}$-dependent $K^+$ channels (100 nM apamine or 20 nM charybdotoxin) did not reproduce the effects of PCMBS on $[Ca^{2+}]_i$ (data not shown).

A human *ether-á-go-go*-related gene-like $K^+$ channel has been described recently in $GH_3$ cells (5). To exclude a possible action of PCMBS on these channels, we measured currents when cells were hyperpolarized to −100 mV from a holding potential of −20 mV in a high-$K^+$ medium in the absence and presence of 50 μM PCMBS. There was no difference between the two conditions.

*Effect of PCMBS on background $Na^+$ conductance.* The effects of PCMBS on the cell input resistance were monitored by injecting hyperpolarizing current pulses (Fig. 5*A*). The mercurial decreased the input resistance from 4.56 ± 1.63 to 2.17 ± 0.86 GΩ (mean ± SE, $n$ = 10; $P < 0.01$, $t$-test). This effect was reversed upon addition of 2 mM DTT. These results indicate that PCMBS increases a membrane conductance. Because $Ca^{2+}$ and $K^+$ currents were not stimulated by the mercurial, the effects on $Na^+$ currents were studied.

Only one of every four cells displayed fast $Na^+$ currents activated by membrane depolarization, and in these cases PCMBS had no effect on the amplitude of the current (data not shown). Simasko (36) described in $GH_3$ cells a background $Na^+$ current (BNC) necessary for spontaneous action potential firing. Figure 6*A* shows that when the membrane potential was clamped at −80 mV, there was an small inward current that decreased when the external $Na^+$ was replaced by NMDG, thus evidencing a BNC. When PCMBS was added to the $Na^+$-containing incubation medium, the inward current increased by −230 pA, and this current was inhibited by removal of external $Na^+$ (replaced by NMDG). The same results were obtained when $Na^+$ was replaced by Tris (data not shown). The effect of PCMBS on $Na^+$ conductance was reversed by DTT (Fig. 6*B*).

Figure 7*A* shows an *I-V* plot of the BNC induced by a 3-s voltage ramp from −100 to +50 mV generated from a holding potential of −30 mV. The currents were measured before (Fig. 7*A*, *trace c*) and after (Fig. 7*A*, *trace d*) treatment with PCMBS. Leak currents were determined in $Na^+$-free medium with (Fig. 7*A*, *trace b*) or without (Fig. 7*A*, *trace a*) PCMBS and subtracted

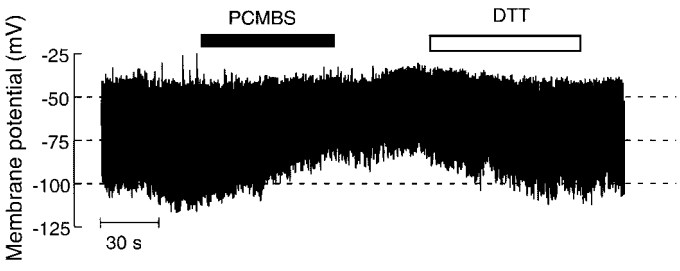


Fig. 5. Effects of PCMBS on input resistance of $GH_3$ cells. Whole cell recording was made in current-clamp mode. Hyperpolarizing current pulses (100 pA, 100 ms) were injected every 2 s. Results are representative of 4 similar experiments.

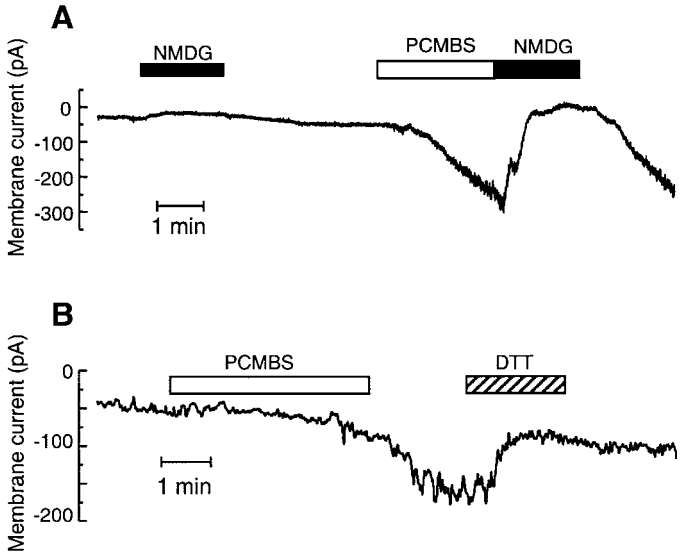


Fig. 6. Effects of PCMBS on whole cell currents in $GH_3$ cells. Whole cell recordings were made in voltage-clamp mode. *A*: the cell was held at −80 mV and perfused with either regular, $Na^+$-containing medium or $Na^+$-free medium [*N*-methyl-D-glucamine (NMDG)] as indicated. Treatment with PCMBS (50 μM) was performed in $Na^+$ medium. Results are representative of 5 similar experiments. *B*: the cell was treated with 50 μM PCMBS in regular, $Na^+$-containing medium and then with the same medium containing DTT as indicated.

from the values obtained in the regular, $Na^+$-containing medium (Fig. 7*B*). The leak current was not modified by PCMBS. Net inward current at −80 mV was −14 ± 3 pA (mean ± SE, $n$ = 9) in the control condition and increased to −67 ± 12 pA after treatment with PCMBS ($P < 0.005$, ANOVA). The slope conductance measured at −80 mV was 259 ± 55 pS in the control condition and 1,675 ± 300 pS after PCMBS. The reversal potential of the background current was about −30 mV, suggesting incomplete specificity for $Na^+$. It is noteworthy that the channel responsible for the BNC of rabbit heart sinoatrial node cells is permeable to $Cs^+$ ions (11). The reversal potential we found in $GH_3$ cells is roughly the mean of the reversal potentials for $Na^+$ and $Cs^+$. The BNC was modified by neither TTX (100 nM) nor amiloride (1 mM) (results not shown).

The increase of BNC by PCMBS can explain the observed membrane depolarization and $Ca^{2+}$ entry (Figs. 1 and 2) by recruitment of voltage-gated $Ca^{2+}$ channels. To confirm this hypothesis, we studied the effects of $Na^+$ on the $[Ca^{2+}]_i$ increase induced by PCMBS in fura 2-loaded $GH_3$ cells. Figure 8 shows that $Na^+$ removal (replaced by NMDG) reverted completely and reversibly the increase of $[Ca^{2+}]_i$. The same trace shows that, after reversal by DTT, a subsequent treatment with PCMBS performed in the absence of $Na^+$ had no effect on $[Ca^{2+}]_i$ and that readdition of $Na^+$ triggered the increase of $[Ca^{2+}]_i$, which could be reverted by DTT. The effect of depolarization with high $K^+$ is also shown in Fig. 8 for comparison.

*PCMBS also increases $[Ca^{2+}]_i$ in rat AP cells in primary culture.* $GH_3$ cells are often used as a model for AP cells, but differences in the behavior of the cell line

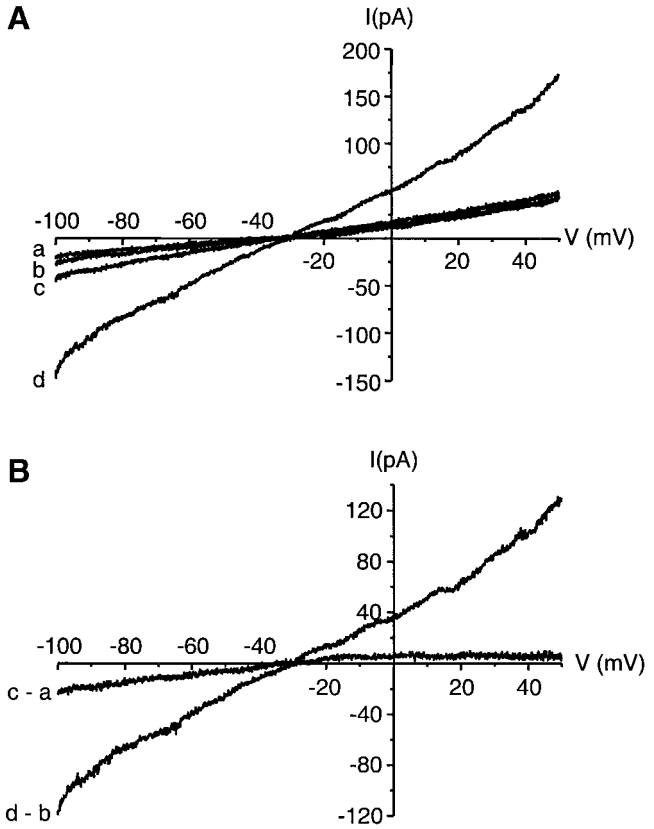


Fig. 7. Effects of PCMBS on $Na^+$ currents in $GH_3$ cells. *A*: *I-V* relationship obtained upon application of a voltage ramp (−100 to +50 mV in 3 s from a holding potential of −30 mV); each trace was averaged from 5 repetitive ramps. Leak currents were recorded in Tris-containing $Na^+$-free medium (*trace a*) and in $Na^+$-free medium plus 50 μM PCMBS (*trace b*). *Trace c* represents the current recorded in the presence of 150 mM NaCl, and *trace d* represents the current recorded after 2 min of treatment with PCMBS. *B*: same currents as in *A* after subtraction of leak currents. Net $Na^+$ currents before (*c* − *a*) and after (*d* − *b*) treatment with PCMBS are shown. Note that leak currents were not modified by PCMBS. Results are representative of 9 similar experiments.

and the primary AP cells are common. For this reason, we tested the effects of PCMBS directly in AP cells in primary culture. Because AP contains at least five well-defined cell subpopulations, each one storing and secreting a different hormone, the effect of PCMBS could differ among the different AP cell types. At the end of the $[Ca^{2+}]_i$ measurements, the cells present in the microscope field were typed by sequential immunocytochemistry of the hormone they store (see METHODS) and the $[Ca^{2+}]_i$ traces were averaged for each cell type. Results are shown in Fig. 9. In lactotropes (Fig. 9*A*, PRL+), PCMBS produced a large increase of $[Ca^{2+}]_i$ that was antagonized by $Na^+$ removal and reversed by DTT. The effect of depolarization with high $K^+$ is also shown for comparison. In somatotropes (Fig. 9*B*, GH+) the effect of the mercurial was also evident, although somewhat slower and weaker. Corticotropes (Fig. 9*C*, ACTH+) showed little or no response. Some of the remaining cells, which must include thyrotropes and gonadotropes, showed a small response. In additional experiments in which thyrotropes and gonadotropes were directly identified (by using antibodies against thyrotropin- and follicle-stimulating hormone, respectively) we found a very small response in thyrotropes ($[Ca^{2+}]_i$ increased from 124 ± 14 to 183 ± 35 nM upon PCMBS application; mean ± SE, $n$ = 8) and no response in gonadotropes ($n$ = 34).

*PCMBS stimulates the same BNC as the GHRH in rat somatotropes.* Measurements of $Na^+$ currents similar to those performed in $GH_3$ cells were carried out in enriched rat somatotropes in primary culture (see METHODS). An inward $Na^+$ current similar to that found in $GH_3$ cells was evidenced in somatotropes (Fig. 10; compare with Fig. 7). The current was measured in $Na^+$-free medium (Fig. 10*A*, *trace a*) and in $Na^+$-containing medium, before (Fig. 10*A*, *trace b*) or after treatment with either 2 nM GHRH (Fig. 10*A*, *trace c*) or 50 μM PCMBS (Fig. 10*A*, *trace d*). GHRH increased the inward $Na^+$ current in three of four cells tested (from 16 ± 7 to 33 ± 6 pA at −80 mV; mean ± SE, $n$ = 3). These results confirm previous observations (19, 21, 29, 37). Treatment with PCMBS produced a much larger increase of the inward $Na^+$ current (to 69 ± 12 pA). The reversal potentials of the inward $Na^+$ currents activated by either GHRH or PCMBS had the same value, nominally about −35 mV (Fig. 10*B*). These results are consistent with both agents stimulating the same current.

## DISCUSSION

We describe here a BNC that can be activated by PCMBS and cause $Ca^{2+}$ entry in pituitary cells. Our results have implications in relation to two different topics: *1*) to explain the toxic effects of mercurials and *2*) to uncover a general mechanism that can regulate the excitability and firing frequency in excitable cells. For the second implication, the silent BNC typical of resting cells should be susceptible to activation by cellular signaling mechanisms. This seems to be the case for somatotropes (see below), but it remains to be studied whether this applies to the BNC present in other excitable tissues.

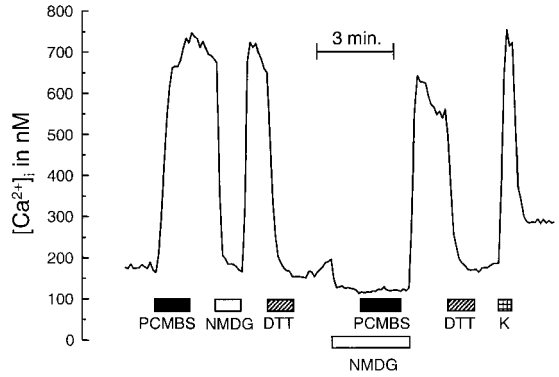


Fig. 8. The increase of $[Ca^{2+}]_i$ induced by PCMBS in $GH_3$ cells is dependent on external $Na^+$. $Na^+$-free medium contained NMDG as the $Na^+$ substitute. An average of 87 cells were present in the same microscope field. The effect of stimulation with high-$K^+$ (50 mM) solution is also shown (K).

A number of studies have identified ionic conductances, mainly $K^+$ and $Ca^{2+}$ channels, as possible targets for the toxic action of mercurials and other sulfhydryl-oxidizing agents (see introduction). However, several effects that cannot be explained by actions on the studied conductances also have been reported. For example, Jungwirth et al. (17) found a membrane depolarization by $Hg^{2+}$ in cultured renal epithelioid (Madin-Darby canine kidney) cells that cannot arise from the increase in $K^+$ conductance they reported. Entry of $Ca^{2+}$ through voltage-gated $Ca^{2+}$ channels induced by $Hg^{2+}$ or methylmercury has been reported in PC-12 cells (31) and in NG108-15 cells (8, 14). Leonhardt et al. (25) found that $Hg^{2+}$ induced an inward current in dorsal root ganglion neurones held at −80 mV, and Arakawa et al. (2) also reported slow inward currents elicited by $Hg^{2+}$ and methylmercury in the same cells. Finally, Marty and Atchison (27) found that methylmercury induced a $Na^+$-dependent $Ca^{2+}$ entry in cerebellar granule neurons. This $Ca^{2+}$ entry was insensitive to TTX, but it could be inhibited by $Ca^{2+}$ channel blockers. The PCMBS-activated BNC described here could explain both the inward current and the increase of $[Ca^{2+}]_i$ reported previously in several tissues. Therefore, this current may be the target responsible for the $Ca^{2+}$ overload and toxicity induced by mercurials in excitable cells. Because the sulfhydryl group responsible for the activation is exposed to the external milieu (see below), the possible involvement of this conductance on the effects of oxidizing agents present in plasma also should be considered.

Treatment with PCMBS increases BNC about sixfold (Figs. 7 and 10), promotes membrane depolarization (Fig. 2), and activates $Ca^{2+}$ entry through L-type voltage-gated $Ca^{2+}$ channels (Figs. 1, 8, and 9). These effects are reversed by DTT, suggesting the involvement of a sulfhydryl group, which should face toward

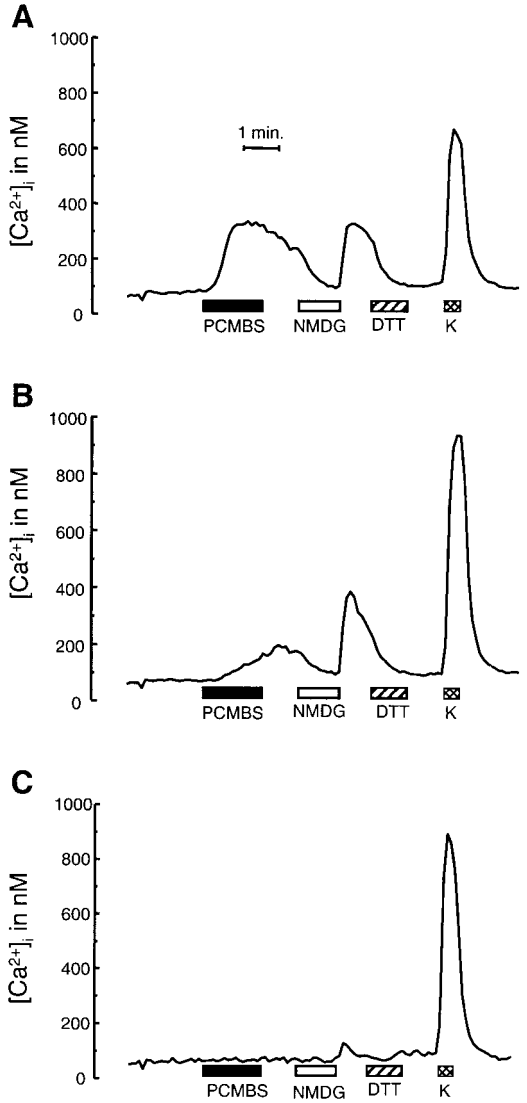


Fig. 9. Effects of PCMBS on $[Ca^{2+}]_i$ in several rat anterior pituitary cell types. At the end of the $[Ca^{2+}]_i$ measurements, the cells present in the microscope field were identified by multiple sequential primary immunocytochemistry by using antibodies against prolactin, growth hormone, and corticotropin (see METHODS). Each trace corresponds to the average of all the cells of every type: lactotropes (*A*; $n$ = 49), somatotropes (*B*; $n$ = 32), and corticotropes (*C*; $n$ = 4). Other details are as described in Figs. 1 and 8.

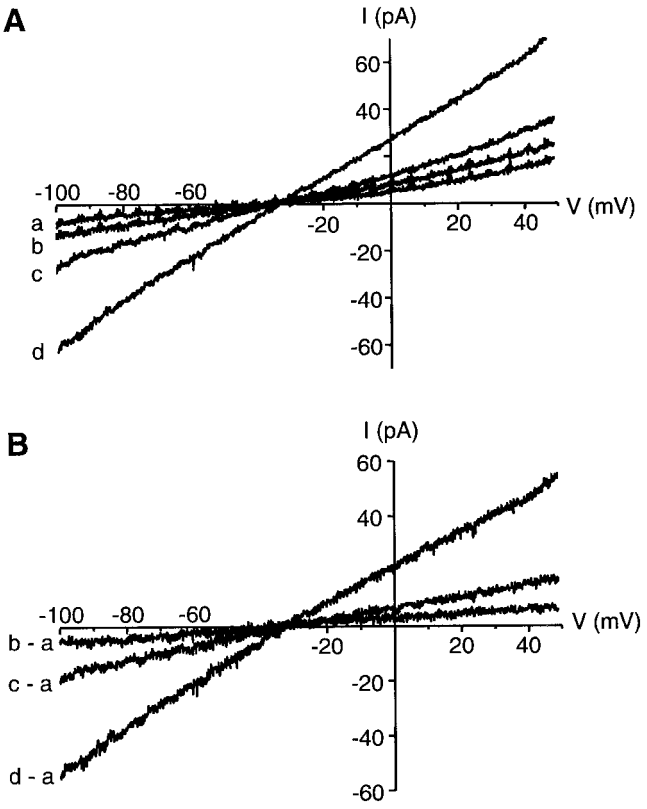


Fig. 10. Growth hormone-releasing hormone (GHRH) and PCMBS elicit an inward $Na^+$ current in rat somatotropes. Enriched rat somatotropes were prepared as described in METHODS. *A*: *I-V* relationships obtained in the same cell in $Na^+$-free medium (*trace a*) or in $Na^+$-containing medium before (*trace b*) or after 3 min of treatment with either 2 nM GHRH (*trace c*) or 50 μM PCMBS (*trace d*). GHRH was washed for 10 min before application of PCMBS. *B*: same currents after subtraction of leak currents; control current ($b - a$) and currents in the presence of GHRH ($c - a$) or PCMBS ($d - a$) are shown. Other details are as described in Fig. 7. Results are representative of 3 similar experiments.

the extracellular medium, since PCMBS is membrane impermeant. This suggestion was confirmed by using membrane-impermeable methanethiosulfonate sulfhydryl reagents. Modulation of ion channel activity through interactions with sulfhydryl groups has been reported for several channels. For example, the cardiac $Na^+$ channel is known to possess a cysteine facing toward the extracellular side that, upon interaction with sulfhydryl reagents, modifies the channel activity (4, 7). This cysteine residue confers insensitivity to TTX (33), which also was found for the BNC described here. The cardiac $Na^+$ channel, however, is gated by membrane depolarization and differs in this from the BNC we found in AP cells.

Simasko and coworkers (32, 36) have identified recently a BNC in pituitary $GH_3$ cells and lactotropes that is necessary for the generation of the spontaneous depolarizations observed in these cells. When this current is eliminated by $Na^+$ removal, the plasma membrane hyperpolarizes, spontaneous firing ceases, $[Ca^{2+}]_i$ decreases, and the basal prolactin secretion is reduced (32, 37). Modulation of BNC by physiological factors would be an efficient mechanism for controlling the firing frequency and, thus, the physiological output of the cell, but there is no information on this topic. Sankaranarayanan and Simasko (32) speculate that an increase of BNC might be the basis for the stimulatory action of GHRH on somatotropes, where GHRH is known to act by stimulating a $Na^+$ conductance (18, 21, 29, 37), but they did not study BNC in this cell type. Here we have found that PCMBS treatment activates a BNC in lactotropes and somatotropes. In somatotropes the reversal potentials of the $Na^+$ currents stimulated by GHRH and PCMBS were the same, suggesting that both agents may activate the same ion channel (Fig. 10). The PCMBS-activated current was very weak in thyrotropes and absent in corticotropes and gonadotropes. Thus BNC seems to be most prominent in the cell types known to show spontaneous electric activity sensitive to modulation by secretagogues (28).

The permeability of the lipid bilayer to ionic species is too low to explain the background conductances of the plasma membranes, suggesting that the ion movements may take place through specific pathways, probably involving pore-forming proteins. However, leak currents are difficult to study because they cannot be activated during electrophysiological protocols. It is clear, though, that changes in these conductances may modify membrane potential and, hence, the electrical activity of excitable cells. BNC could be the target of extracellular or intracellular messengers, as suggested by our results in somatotropes. In addition, a second reading of the reported effects of mercurials (see above) is suggestive of the presence of BNC in several excitable cells. Treatment with PCMBS offers an easy procedure to detect BNC in a given cell type. Work is in progress to screen the presence of BNC in different tissues and to investigate the molecular substrate of this current.

This work was supported by Spanish Dirección General de Enseñanza Superior Grant PB97-0474. C. Villalobos was supported by a fellowship from the Spanish Ministerio de Educación y Cultura. Antisera to pituitary hormones were a gift from the National Hormone and Pituitary Program, the National Institute of Diabetes and Digestive and Kidney Diseases, the National Institute of Child Health and Human Development, and the US Department of Agriculture.

## REFERENCES

1. **Akabas MH, Stauffer DA, Xu M, and Karlin A.** Acetylcholine receptor channel structure probed in cysteine-substitution mutants. *Science* 258: 307–310, 1992.
2. **Arakawa O, Nakahiro M, and Narahashi T.** Mercury modulation of GABA-activated chloride channels and non-specific cation channels in rat dorsal root ganglion neurons. *Brain Res* 551: 58–63, 1991.
3. **Atchison WD and Hare MF.** Mechanisms of methylmercury-induced neurotoxicity. *FASEB J* 8: 622–629, 1994.
4. **Backx PH, Yue DT, Lawrence JH, Marban E, and Tomaselli GF.** Molecular localization of an ion-binding site within the pore of mammalian sodium channels. *Science* 257: 248–251, 1992.
5. **Barros F, Del Camino D, Pardo L, Palomero T, Giraldez T, and De La Peña P.** Demonstration of an inwardly rectifying $K^+$ current component modulated by thyrotropin-releasing hormone and caffeine in $GH_3$ rat anterior pituitary cells. *Pflügers Arch* 435: 119–129, 1997.
6. **Caputo C, Perozo E, and Bezanilla F.** Chemical modification of squid axon $K^+$ channel-SH groups with the organic mercurial compound *p*-hydroxymercuriphenylsulfonic acid (PHMPS). *Pflügers Arch* 428: 315–322, 1994.
7. **Chiamvimonvat N, O'Rourke B, Kamp TJ, Kallen RG, Hofmann F, Flockerzi V, and Marban E.** Functional consequences of sulfhydryl modification in the pore-forming subunits of cardiovascular $Ca^{2+}$ and $Na^+$ channels. *Cardiovasc Res* 76: 325–334, 1994.
8. **Denny M and Atchison WD.** Mercurial-induced alterations in neuronal divalent cation homeostasis. *Neurotoxicology* 17: 47–61, 1996.
9. **De Weille JR, Schweitz H, Maes P, Tartar A, and Lazdunski M.** Calciseptine, a peptide isolated from black mamba venom, is a specific blocker of L-type calcium channel. *Proc Natl Acad Sci USA* 88: 2437–2440, 1991.
10. **Dubinsky JM and Oxford GS.** Ionic currents in two strains of rat anterior pituitary tumor cells. *J Gen Physiol* 83: 309–339, 1984.
11. **Hagiwara N, Irisawa H, Kasanuki H, and Hosoda S.** Background current in sino-atrial node cells of the rabbit heart. *J Physiol (Lond)* 448: 53–72, 1992.
12. **Hamill O, Marty A, Neher E, Sakmann B, and Sigworth F.** Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch* 391: 85–100, 1981.
13. **Han J, Kim E, Ho W, and Earm Y.** Sulfhydryl redox modulates ATP-sensitive $K^+$ channels in rabbit ventricular myocytes. *Biochem Biophys Res Commun* 219: 900–903, 1996.
14. **Hare MF, McGinnis KM, and Atchison WD.** Methylmercury increases intracellular concentrations of $Ca^{2+}$ and heavy metals in NG108–15 cells. *J Pharmacol Exp Ther* 266: 1626–1635, 1993.
15. **Horn R and Marty A.** Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *J Gen Physiol* 92: 145–159, 1988.
16. **Islam M, Berggren P, and Larsson O.** Sulfhydryl oxidation induces rapid and reversible closure of the ATP-regulated $K^+$ channel in the pancreatic beta-cell. *FEBS Lett* 319: 128–132, 1993.
17. **Jungwirth A, Ritter M, Paulmichl M, and Lang F.** Activation of cell membrane potassium conductance by mercury in cultured renal epithelioid (MDCK) cells. *J Cell Physiol* 146: 25–33, 1991.
18. **Kato MJ, Hoyland J, Sikdar SK, and Mason WT.** Imaging of intracellular calcium in rat anterior pituitary cells in response to

growth hormone releasing factor. *J Physiol (Lond)* 447: 171–189, 1992.
19. **Kato M and Sakuma Y.** Regulation by growth hormone-releasing hormone and somatostatin of a $Na^+$ current in the primary cultured rat somatotroph. *Endocrinology* 138: 5096–5100, 1997.
20. **Krippeit-Drews P, Zempel G, Ammon HP, Lang F, and Drews G.** Effects of membrane-permeant and -impermeant thiol reagents on $Ca^{2+}$ and $K^+$ channel currents of mouse pancreatic β cells. *Endocrinology* 136: 464–467, 1994.
21. **Kwiecien R, Robert C, Cannon R, Vigues S, Arnoux A, Kordon C, and Hammond C.** Endogenous pacemaker activity of rat tumour somatotrophs. *J Physiol (Lond)* 508: 883–905, 1998.
22. **Lacampagne A, Duittoz A, Bolaños P, Peineau N, and Argibay JA.** Effect of sulfhydryl oxidation on ionic and gating currents associated with L-type calcium channels in isolated guinea-pig ventricular myocytes. *Cardiovasc Res* 30: 799–806, 1995.
23. **Leech CA and Habener JF.** A role for $Ca^{2+}$-sensitive nonselective cation channels in regulating the membrane potential of pancreatic β-cells. *Diabetes* 47: 1066–1073, 1998.
24. **Leech CA, Holz CG, and Habener JF.** Signal transduction of PACAP and GLP-1 in pancreatic β-cells. *Ann NY Acad Sci* 805: 81–93, 1996.
25. **Leonhardt R, Pekel M, Platt B, Hass H, and Büsselberg D.** Voltage-activated calcium channel currents of rat DRG neurons are reduced by mercury chloride ($HgCl_2$) and methylmercury ($CH_3HgCl$). *Neurotoxicology* 17: 85–92, 1996.
26. **López MG, Villarroya M, Lara B, Martínez-Sierra R, Albillos A, García AG, and Gandía L.** Q- and L-type $Ca^{2+}$ channels dominate the control of secretion in bovine chromaffin cells. *FEBS Lett* 349: 331–337, 1994.
27. **Marty M and Atchison WD.** Pathways mediating $Ca^{2+}$ entry in rat cerebellar granule cells in vitro exposed to methylmercury. *Toxicol Appl Pharmacol* 147: 319–330, 1997.
28. **Mollard P and Schlegel W.** Why are endocrine pituitary cells excitable? *Trends Endocrinol Metab* 10: 361–365, 1996.
29. **Naumov AP, Herrington J, and Hille B.** Actions of growth hormone-releasing hormone on rat pituitary cells: intracellular calcium and ionic currents. *Pflügers Arch* 427: 414–421, 1994.
30. **Ritchie AK.** Two distinct calcium-activated potassium currents in a rat anterior pituitary cell line. *J Physiol (Lond)* 385: 591–609, 1997.
31. **Rossi AD, Larsson O, Manzo L, Orrenius S, Vahter M, Berggren PO, and Nicotera P.** Modifications of $Ca^{2+}$ signalling by inorganic mercury in PC12 cells. *FASEB J* 7: 1507–1514, 1993.
32. **Sankaranarayanan S and Simasko SM.** A role for background sodium current in spontaneous action potentials and secretion from rat lactotrophs. *Am J Physiol Cell Physiol* 271: C1927–C1934, 1996.
33. **Satin J, Kyle JW, Chen M, Bell P, Cribbs LL, Fozzard HA, and Rogart RB.** A mutant of TTX-resistant cardiac sodium channels with TTX-sensitive properties. *Science* 256: 1202–1205, 1992.
34. **Shafer TJ and Atchison WD.** Methylmercury blocks N- and L-type $Ca^{2+}$ channels in nerve growth factor-differentiated pheochromocytoma (PC12) cells. *J Pharmacol Exp Ther* 258: 149–157, 1991.
35. **Simasko SM.** Reevaluation of the electrophysiological actions of thyrotropin-releasing hormone in a rat pituitary cell line ($GH_3$). *Endocrinology* 124: 2015–2026, 1991.
36. **Simasko SM.** A background sodium conductance is necessary for spontaneous depolarizations in rat pituitary cell line $GH_3$. *Am J Physiol Cell Physiol* 266: C709–C719, 1994.
37. **Takano K, Takei T, Teramoto A, and Yamashita N.** GHRH activates a nonselective cation current in human GH-secreting adenoma cells. *Am J Physiol Endocrinol Metab* 270: E1050–E1057, 1996.
38. **Villalobos C, Fonteriz RI, López MG, García AG, and García-Sancho J.** Inhibition of voltage-gated $Ca^{2+}$ entry into $GH_3$ and chromaffin cells by imidazole antimycotics and other cytochrome P450 blockers. *FASEB J* 6: 2742–2747, 1992.
39. **Villalobos C and García-Sancho J.** Glutamate increases cytosolic calcium in $GH_3$ pituitary cells acting via a high-affinity glutamate transporter. *FASEB J* 9: 815–819, 1995.
40. **Villalobos C and García-Sancho J.** Capacitative $Ca^{2+}$ entry contributes to $Ca^{2+}$ entry induced by thyrotropin-releasing hormone (TRH) in $GH_3$ pituitary cells. *Pflügers Arch* 430: 923–935, 1995.
41. **Villalobos C, Nuñez L, Frawley LS, García-Sancho J, and Sanchez A.** Multi-responsiveness of single anterior pituitary cells to hypothalamic releasing hormones: a cellular basis for paradoxical secretion. *Proc Natl Acad Sci USA* 94: 14132–14137, 1997.
42. **Villalobos C, Nuñez L, and García-Sancho J.** Functional glutamate receptors in a subpopulation of anterior pituitary cells. *FASEB J* 10: 654–660, 1996.
43. **Villalobos C, Nuñez L, and García-Sancho J.** Mechanisms for stimulation of anterior pituitary cells by arginine and other amino acids. *J Physiol (Lond)* 502: 421–431, 1997.