Role of Intracellular pH in Secretion from Adrenal Medulla Chromaffin Cells

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The role of intracellular pH in stimulus-secretion coupling was investigated in cultured bovine adrenal medullary chromaffin cells. NH₄Cl (1-25 mM) did not affect basal catecholamine or ATP release but markedly inhibited nicotine- or high K⁺-induced release by up to 60%. The inhibition had a rapid onset (<1 min) and was maximal at about 5 mM NH₄Cl. The effect of NH₄Cl was largely sustained over 20 min and was reversed upon NH₄Cl removal. Sodium propionate did not affect secretion but partially reversed the inhibition by NH₄Cl in a concentration-dependent manner. Methylamine (10 mM) produced a similar, but slower, inhibition than NH₄Cl. Monensin (1-10 μM) inhibited catecholamine secretion by 30-60%, and its effect was reduced in the presence of NH₄Cl. Using the fluorescent Ca²⁺ probe Fura-2, we found that the increase of [Ca²⁺], following stimulation was not altered by concentrations of NH₄Cl which inhibited secretion maximally. Measurement of cytosolic pH (pHₗ) with the fluorescent probe 2',7'-bis-carboxyethyl-5(6)-carboxyfluorescein (BCECF) revealed an alkalization by NH₄Cl (2.5-25 mM) of 0.1-0.23 pH units and acidification by sodium propionate (10-20 mM) of 0.2-0.25 pH units, with intermediate combined effects. Monensin (1 μM) caused a cytosolic acidification of 0.26 pH units. All pH changes were partly recovered in 15 min. Fluorescence quenching measurements using the weakly basic fluorescent probe acridine orange indicated the accumulation of the probe into acidic compartments, presumably the chromaffin granules, which was strongly reduced by both NH₄Cl and monensin. From these findings we conclude that the pH of the chromaffin granule modulates secretion by affecting some step in the secretory process unrelated to the rise in [Ca²⁺].

The response of most secretory cells to secretory stimuli has been shown to be a transient rise in intracellular pH (pHₗ) (6-8). Although this alkalization is secondary to an initial acidification caused by the increase in [Ca²⁺], the results of these studies suggest that pH may be an important factor in regulating the secretory response.

The role of pH in the secretion of chromaffin granules from adrenal chromaffin cells has received much attention in recent years (9-12). Chromaffin granules store large amounts of catecholamines (500 mM), ATP (122-150 mM), chromogranins (120 mg/ml), enkephalins, Ca²⁺, and other substances and are secreted upon cholinergic receptor activation or membrane depolarization by exocytosis. The pH inside the granules is acidic (~5.5) due to the acidic granule proteins and the pumping of protons into the granule by the granule membrane H⁺-ATPase (13). The low internal pH appears to be essential for the storage of biogenic amines and for catecholamine transport (12) but may also be more directly related to secretion. In the chemiosmotic hypothesis for secretion formulated by Pollard et al. (9), an electrochemical gradient for protons generated by the H⁺-ATPase in the granule membrane is responsible for the anion-dependent lysis of isolated granules and possibly the release of granules in intact chromaffin cells. However, studies on the effects of pHₗ, pHₘ, the H⁺-ATPase activity, and the pH gradient across the granule membrane on secretion, which have been carried out in both intact and permeabilized chromaffin cells, have not revealed a distinct role for either pHₗ or ΔpH across the granule membrane in secretion (10-12).

In this study, we report an inhibitory effect of the permeant weak bases, NH₄Cl and methylamine, and of the Na⁺-H⁺-ionophore monensin on stimulated catecholamine and ATP secretion from bovine adrenal chromaffin cells. We provide evidence that this inhibition is not the result of an inhibitory action on intracellular Ca²⁺ levels but that weak bases and monensin inhibit secretion by alkalizing an essential acidic cell compartment, which is probably the secretory chromaffin granule.

EXPERIMENTAL PROCEDURES

Cell Preparation and Culture—Bovine adrenal glands were obtained from the slaughterhouse and kept on ice for ~90 min. The glands were warmed and perfused three times with Ca²⁺- and Mg²⁺-free Locke's solution containing 154 mM NaCl, 5.6 mM KCl, 5 mM NaHCO₃, 5.6 mM glucose, and 5 mM HEPES (pH 7.4, 37 °C). The medullary tissue was dissociated by three successive perfusions with sterile Ca²⁺- and Mg²⁺-free Locke's solution containing 0.5% BSA (Sigma, type V), 0.2, 0.1, and 0.1% collagenase (Sigma, type IA) for each 15 min at 37 °C. The medullae were then removed, minced, and cooled.

The abbreviations used are: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BCECF (AM), 2',7'-bis-carboxyethyl-5(6)-carboxyfluorescein (acetoxymethyl ester); AO, acridine orange; MES, 4-morpholinoethanesulfonic acid; BSA, bovine serum albumin; EGTA, [ethylenebis(oxyethylenetetrol)]tetraacetate acid.
in Locke's solution, and filtered through nylon gauze (mesh size 60 μm). Filtered cells were pelleted and washed twice in Locke's solution. The filtrate was digested for an additional 30 min with 0.1% collagenase. This second digest was filtered, and the cells were washed and combined with the cells from the first digestion step.

Chromaffin cells were purified on a Percoll gradient. A Percoll solution was prepared by diluting 9 volumes of Percoll (Pharmacia LKB Biotechnology Inc.) with 1 volume of 10 times concentrated Locke's solution. The Percoll solution was centrifuged at 20,000 g for 20 min, and the cells were removed from the bottom layer of red blood cells by pipetting. The Percoll was removed by washing in Ca²⁺-containing Locke's solution. The cells were suspended in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium, buffered with 15 mM HEPES (Sigma) and 21 mM NaHCO₃, and containing 5% heat-inactivated fetal bovine serum (Sigma), penicillin (100 units/mL), streptomycin (100 μg/mL), and Fungizone (250 ng/mL). Cells were cultured at densities of 0.5-10⁻²-2·10⁻⁴/cm² and 1.5-10⁶-2·10⁶/cm² in culture medium (Costar 3075), multiwell culture plates (Costar 3524), or bacterial dishes (Falcon 1029). Chromaffin cells cultured in the bacterial dishes did not attach to the dish but remained in suspension.

Catecholamine Release Experiments—After 2-4 days cells were harvested from flasks or dishes by shaking or pipetting and used for experiments. Cells were suspended in the Percoll gradient on a shaker and washed twice in Locke's solution. The cell suspension was mixed with the Percoll gradient and centrifuged for 20 min at 20,000 g (15 °C). The bottom layer of red blood cells was removed. The cells were resuspended in 0.7 ml of medium and stored on ice to minimize ATP release from the cells.

Experiments were done at room temperature. Continuous monitoring of BCECF fluorescence was carried out in a Spex Fluorolog spectrofluorometer (Spex Industries, Metuchen, NJ) equipped with a digital plotter and a microprocessor (DM1B). Excitation and emission wavelengths were 500 and 550 nm, and 1-2 nm slits were used, respectively. A 524-nm cutoff long pass filter was used across the emission path to reduce scattered light. For the recording small aliquots containing approximately 3·5·10⁻⁹ BCECF-loaded cells were transferred into acrylic cuvettes containing 2.5 ml of medium at room temperature. Cells in the cuvette were gently stirred and allowed to equilibrate for ~5 min, at which time the background fluorescence became stable.

Calibration of pH, versus fluorescence was carried out at the end of each experiment by disrupting the cells with the nonionic detergent C₁₀E₄₂ (nonaethylene glycolcethal ether, Calbiochem, 1 mM), followed by titration of the medium with small aliquots of MES (1 M, pH 6.0). BCECF fluorescence varies linearly with pH in the range 6.4-7.7 (16), and calibration could therefore be done using a linear plot of fluorescence intensity versus medium pH. No correction was introduced for any possible red shift that might occur with fluorescein derivatives trapped in chromaffin cells, as has been observed in other systems (16). However, a correction was made for fluorescence from BCECF present in the extracellular medium during the experiment. For this purpose, we used anti-fluorescein antibodies (gift from Dr. A. Sztutin, University of Chile, Santiago, Chile) (17, which have been reported to be effective quenchers of BCECF fluorescence (18). Addition of these antibodies to intact BCECF-loaded cells showed that a minor part of the signal was indeed due to BCECF present in the extracellular medium. Assuming that BCECF fluorescence varies linearly with medium pH at a fixed probe concentration and, conversely, that fluorescence varies linearly with probe concentration at a given pH, we corrected both experimental data and calibration points for extracellular BCECF fluorescence quenched by the antibodies and converted the data off-line from fluorescence units to pH units.

Measurement of Intracellular Ca²⁺—The procedure for loading chromaffin cells with the membrane-permeant ester Fura-2-AM (Molecular Probes, Eugene, OR) (5 μM) (19) was similar to the procedure used with BCECF-AM, with the following modifications: 1) the loading time was increased to 60 min; 2) the background level of fluorescence was used with BCECF-AM, with the following modifications: 1) the loading time was increased to 60 min; 2) the background level of fluorescence was omitted, as it did not appear to affect the cell responses to secretagogues. The cells were washed twice, first with medium containing 1% BSA and then with BCECF-free medium. The final aliquot of Fura-2-loaded cells was kept on ice during the course of the experiments.

For Fura-2 fluorescence recording, the excitation and emission wavelengths were set at 340 and 510 nm, respectively, and a 434-nm cutoff low pass filter was used across the emission path. Calibration of [Ca²⁺], versus fluorescence was carried out as follows: at the end of each experiment, detergent (C₁₀E₄₂, 1 mM) was added to the cells released the probe into the medium. This allowed the (saturating) fluorescence of Fura-2 (Fₛ) to be recorded. Next, a small aliquot (50 μl) of a mixture of 0.2 M EGTA and 1 M Tris (pH 10.2) was added to the cuvette to reduce the Ca²⁺ concentration to subnanomolar levels, thus allowing the minimum Fura-2 fluorescence (Fₐ) to be recorded. Fluorescence intensities (F) were converted to Ca²⁺ concentrations using the calibration equation for single wavelength excitation measurements,

\[ [Ca^{2+}] = k_d (F - F_{\text{min}})/(F_{\text{max}} - F), \]

where \( k_d \) is the dissociation constant of the Ca²⁺-Fura-2 complex which was taken to be 224 nM (10). The cell autofluorescence was subtracted from all the data, and the curve was recorded using the procedure described above.

Acridine Orange Fluorescence Measurements—Cells from tissue culture flasks were washed twice and resuspended in Krebs solution containing 0.5% BSA (8·10⁻⁹ cells/mL). Loading of the cells with the

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AO was carried out by adding 100 µl of cell suspension to a cuvette with 2.5 ml of medium containing 1 µg/ml AO (Sigma) at room temperature. The uptake of AO was monitored using a Spex Fluorolog spectrofluorometer by measuring the fluorescence quenching of AO, which indicates accumulation of the weakly basic dye into acidic compartments. Excitation and emission wavelengths were 450 and 500 nm, and 1- and 2-mm slits were used, respectively. Equilibrium was reached after 20-30 min of incubation. The effect of NH₄Cl and monensin on AO fluorescence was determined by adding concentrated aliquots of these substances to the cuvette and recording the fluorescence change. The fluorescence of AO was not significantly dependent on pH in the relevant range (5.8-7.4), and pH changes were reflected solely by the influx or efflux of AO in the acidic compartments resulting in a change in AO fluorescence quenching. The semiquantitative results are expressed in arbitrary fluorescence units.

**RESULTS**

Effect of Weak Bases on Catecholamine Secretion—Chromaffin cells, from either suspended or plated cultures, had a variable secretory response to nicotine (15 µM), high external K⁺ (25-50 mM), or external Ba²⁺ (3 mM). Using nicotine (15 µM) as a stimulant, suspended cells secreted 1-12%, while plated cells secreted 5-20% of their total catecholamine content within 5 min. Exposure to high external K⁺ (50 mM) induced a 2.5-10% release in suspended cells and a 7.5-13% release in plated cells within 5 min. Ba²⁺ (3 mM) treatment resulted in a slower but more massive release, i.e. approximately 50% of total catecholamine in 20 min in plated cells.

Preincubation of the cells with NH₄Cl consistently inhibited both nicotine- and high K⁺-stimulated catecholamine secretion. The inhibition occurred regardless of the extent of stimulated secretion in both plated and suspended cells. The inhibition by NH₄Cl had a rapid onset (<20 s) and, for 10 mM NH₄Cl, was maximal after ~0.5-2 min of preincubation with NH₄Cl (Fig. 1A). The extent of the inhibition by 10 µM NH₄Cl slowly diminished with longer preincubation times. A typical inhibition of 50% of the catecholamine released by 15 µM nicotine (5 min) occurred after 2 min of preincubation with NH₄Cl (10 mM), which decreased to ~40% with preincubation times longer than 10 min (Fig. 1A and Table I).

When K⁺ (50 mM) was used as a stimulant, NH₄Cl had a similar inhibitory effect, i.e. 45% inhibition after 1 min of preincubation (Table I). The inhibitory effect of NH₄Cl concentration-dependent and ranged between 8 and 60% inhibition (1 min of preincubation) for nicotine (15 µM)-stimulated secretion at concentrations between 0.5 and 25 mM (Fig. 1B).

Cells preincubated with methylamine also had a diminished secretory response to nicotine and high external K⁺. However, the onset of the inhibition was much slower and the preincubation time needed to attain maximal inhibition was much longer than with NH₄Cl (Fig. 1A). Partial recovery from the inhibition in time, as seen with NH₄Cl (10 mM), was not observed with methylamine (10 mM) (Fig. 1A). Maximal inhibition by methylamine (10 mM) was 43% for nicotine (15 µM)-stimulated secretion but was only attained after 20 min of preincubation (Table I). Methylamine (10 mM) also inhibited high K⁺-induced secretion, and the inhibition was 32% after 20 min of preincubation (Table I). In the absence of secretagogues, both NH₄Cl and methylamine did not cause any significant change in the basal release of catecholamines, which was 0.5-1% (per 5 min) of the total catecholamine content of the cells.

The inhibitory effect of NH₄Cl was rapidly and completely reversed upon removal of the NH₄Cl. When cells were preincubated with 10 mM NH₄Cl for 5 min and then transferred to a medium without NH₄Cl, the secretory response to nicotine (15 µM) was completely restored from 46 ± 6% (5 min of preincubation with NH₄Cl) to 110 ± 7% of the previous control within 20 s after the removal of the NH₄Cl (means ± S.E. of five experiments).

Preincubation with NH₄Cl also inhibited Ba²⁺-induced catecholamine secretion although the effect was smaller, i.e. a maximal inhibition of 19% was obtained with 10 mM NH₄Cl (Table I). The secretion induced by 100 µM veratridine was, surprisingly, enhanced 2.55-fold with 10 mM NH₄Cl and slightly increased by 18% with 10 mM methylamine (Table I).

**Effects of Weak Base on ATP Release**—The release of ATP stimulated by either nicotine or high K⁺ was also inhibited by pretreatment with NH₄Cl. The inhibition had a rapid onset and was dependent on the NH₄Cl concentration (Fig. 2A). The extent of the inhibition of ATP release apparently was larger than the inhibition of catecholamine release. As shown in Fig. 2A, the percentage of ATP released from the cells by 15.5 µM nicotine was 0.71% and was inhibited by NH₄Cl to 28% (10 mM) and 9% (25 mM) of this value. On the average, 10 mM NH₄Cl (10-60 s of preincubation) inhibited nicotine (15.5 µM)-stimulated ATP release (average ± S.E. of five
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**TABLE I**

<table>
<thead>
<tr>
<th>Secretagogue</th>
<th>Control</th>
<th>NH4Cl (10 mM)</th>
<th>Methylamine (10 mM)</th>
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<tr>
<td></td>
<td>% of total (n)</td>
<td>min</td>
<td>% of control (n)</td>
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<tr>
<td>Nicotine (15 μM, 4 min)</td>
<td>8.5 ± 1 (22)</td>
<td>1</td>
<td>57 ± 2 (5)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>51 ± 6 (5)</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>60 ± 7 (7)</td>
<td>20</td>
</tr>
<tr>
<td>High K⁺ (50 mM, 5 min)</td>
<td>8.4 ± 1 (7)</td>
<td>1</td>
<td>55 ± 6 (7)</td>
</tr>
<tr>
<td>Ba⁺⁺ (3 mM, 20 min)</td>
<td>50 ± 6 (4)</td>
<td>1</td>
<td>91 ± 2 (4)</td>
</tr>
<tr>
<td>Veratridine (100 μM, 15 min)</td>
<td>4 ± 1 (4)</td>
<td>10</td>
<td>255 ± 39 (4)</td>
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**TABLE II**

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Catecholamine release</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of control (n)</td>
</tr>
<tr>
<td>Control</td>
<td>100 ± 4 (12)</td>
</tr>
<tr>
<td>NH4Cl (10 mM)</td>
<td>54 ± 4 (12)</td>
</tr>
<tr>
<td>Sodium propionate</td>
<td></td>
</tr>
<tr>
<td>5 mM</td>
<td>106 ± 9 (2)</td>
</tr>
<tr>
<td>10 mM</td>
<td>109 ± 9 (4)</td>
</tr>
<tr>
<td>20 mM</td>
<td>104 ± 12 (5)</td>
</tr>
<tr>
<td>NH4Cl/sodium propionate</td>
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</tr>
<tr>
<td>10/5 mM</td>
<td>63 ± 8 (2)</td>
</tr>
<tr>
<td>10/10 mM</td>
<td>69 ± 8 (4)</td>
</tr>
<tr>
<td>10/20 mM</td>
<td>71 ± 4 (5)</td>
</tr>
<tr>
<td>Monensin</td>
<td></td>
</tr>
<tr>
<td>1 μM</td>
<td>77 ± 3 (5)</td>
</tr>
<tr>
<td>10 μM</td>
<td>38 ± 3 (4)</td>
</tr>
<tr>
<td>NH4Cl/monensin</td>
<td></td>
</tr>
<tr>
<td>10 mM/1 μM</td>
<td>56 ± 3 (5)</td>
</tr>
<tr>
<td>10 mM/10 mM</td>
<td>35 ± 3 (4)</td>
</tr>
</tbody>
</table>

*100% = 10.2 ± 0.9% of total catecholamine content (mean ± S.E.; n = 12).

Fig. 2. Time course of ATP release from chromaffin cells in the presence and absence of NH4Cl. A, ATP release was stimulated by 15.5 μM nicotine at t = 0 after 10 s of preincubation with 0, 10, or 25 mM NH4Cl. ATP was measured on-line as described under “Experimental Procedures.” B, ATP release was stimulated by 3.1, 9.3, or 15.5 μM nicotine at t = 0 without NH4Cl. The results were plotted as a percentage of total ATP present in the cells at the end of the experiment. Results are typical for three experiments.

Experiments: 1.39 ± 0.23%) to 39 ± 9% of the control (mean ± S.E., n = 5), in contrast to 57 ± 2% for catecholamine release (Table I), and 25 mM NH4Cl inhibited ATP release to 31 ± 9% of the control (mean ± S.E., n = 3).

When lower concentrations (<15.5 μM) of nicotine were used, ATP release was also reduced but with a different kinetic pattern (Fig. 2B). The release with 9.3 and 3.1 μM nicotine averaged 69 ± 13% and 13 ± 4% of the control release with 15.5 μM nicotine, respectively (results ± S.E. of five and three experiments). NH4Cl did not significantly change the luminescence of the luciferin-luciferase mixture (results not shown).

By fitting the ATP release induced by nicotine (15 μM) plus various concentrations of NH4Cl (Fig. 2A) to a single exponential function, the inhibitory effect of NH4Cl could be expressed by a change in t, the time constant for the release. Under normal conditions, τ was found to be 15.2 ± 0.7 s (n = 9, mean ± S.E.) and decreased to 8.70 ± 1.3 s (n = 7) with 10 mM NH4Cl and to 4.8 ± 0.6 s (n = 3) with 25 mM NH4Cl. When the nicotine concentration was lowered (Fig. 2B), τ increased from 15.2 ± 0.7 at 15.5 μM nicotine to 19.2 ± 1.8 at 9.3 μM nicotine (n = 5) and 29.4 ± 0.9 at 3.1 μM nicotine (n = 3).

Also in the case of ATP release, removal of NH4Cl resulted in an immediate return to a normal secretory response.

Effect of NH4Cl, Sodium Propionate, and Monensin on Catecholamine Secretion—In order to examine whether the inhibition of secretion by weak bases was related to pH, we investigated the effect of sodium propionate and the combined effect of NH4Cl (10 mM) and various sodium propionate concentrations on catecholamine secretion. Preincubation with sodium propionate alone for 1 min slightly increased the subsequent response of the cells to nicotine, (15 μM), and simultaneous preincubation with both NH4Cl (10 mM) and...
sodium propionate (5–20 mM) for 1 min inhibited secretion to a lesser degree than NH4Cl alone (Table II). For example, with 10 mM NH4Cl plus 20 mM propionate, the inhibition is 29% compared to 46% for 10 mM NH4Cl alone.

In the absence of secretagogue, both NH4Cl (10 mM) and sodium propionate (20 mM) and their combination had no significant effect on basal catecholamine release. The effect of adding up to 30 mM osmotically active salt solution (NH4Cl/sodium propionate) was considered negligible since 30 mM NaCl did not affect the stimulatory response of the cells.

The nonrheogenic Na+-H+-ionophore monensin also inhibited nicotine-stimulated catecholamine secretion in a concentration-dependent manner (Table II). When monensin was applied in combination with NH4Cl (10 mM) the resulting secretory inhibition was similar to that with NH4Cl alone (at 1 μM monensin) or with monensin (at 10 μM monensin) alone and appeared to be set by whichever substance gave the largest inhibition.

**TABLE III**

<table>
<thead>
<tr>
<th>Incubation</th>
<th>n</th>
<th>Resting Ca(^{2+}) level (nM)</th>
<th>Increase of Ca(^{2+}) level after stimulation</th>
<th>Peak</th>
<th>1 min</th>
<th>Steady state</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5</td>
<td>133 ± 23</td>
<td>255 ± 28</td>
<td>174 ± 22</td>
<td>78 ± 5</td>
<td></td>
</tr>
<tr>
<td>NH4Cl</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mM</td>
<td>4</td>
<td>145 ± 11</td>
<td>256 ± 19</td>
<td>171 ± 14</td>
<td>70 ± 5</td>
<td></td>
</tr>
<tr>
<td>25 mM</td>
<td>2</td>
<td>193</td>
<td>343 ± 26</td>
<td>264 ± 22</td>
<td>97</td>
<td></td>
</tr>
<tr>
<td>Sodium propionate (20 mM)</td>
<td>4</td>
<td>107 ± 3</td>
<td>203 ± 21</td>
<td>84 ± 6</td>
<td>54 ± 2</td>
<td></td>
</tr>
</tbody>
</table>

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**Determination of Intracellular Ca\(^{2+}\) Concentrations**—Secretagogues such as acetylcholine, nicotine, or high K\(^+\) are known to induce a rise in intracellular Ca\(^{2+}\) concentration in chromaffin cells (20), and it is thought that this Ca\(^{2+}\) acts as a second messenger in secretion. In an attempt to find out whether or not the inhibitory effect of weak bases was through a mechanism which altered intracellular Ca\(^{2+}\), we investigated the effect of NH4Cl on the intracellular Ca\(^{2+}\) response to high K\(^+\) using the fluorescent Ca\(^{2+}\) indicator probe, Fura-2.

The results of the measurements with Fura-2 are shown in Fig. 3 and Table III. The resting Ca\(^{2+}\) concentration in our chromaffin cells was 133 ± 23 nM (mean ± S.E., five experiments). Following high K\(^+\) (37.5 mM) stimulation, the Ca\(^{2+}\) level was increased by ~190 nM under control conditions (Fig. 3A). 10 mM NH4Cl had no significant effect on this peak value (Fig. 3B), 25 mM NH4Cl increased the value, and 20 mM sodium propionate decreased it (Fig. 3C). Table III shows that after 30–100 s of preincubation, the resting intracellular Ca\(^{2+}\) concentration was somewhat increased with NH4Cl (10–25 mM) and decreased with sodium propionate (20 mM). Stimulation with 37.5 mM K\(^+\) increased the Ca\(^{2+}\) level by 255
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Fig. 5. Stimulated catecholamine secretion from chromaffin cells as a function of intracellular pH. Data shown are from experiments with: △, ○, various preincubation times with 10 mM NH4Cl; •, 1-min preincubation with various concentrations of NH4Cl; △, V, various preincubation times with 10 mM methylamine and 1-min preincubation with: △, 10 or 20 mM sodium propionate; △, 10 mM NH4Cl plus 10 or 20 mM sodium propionate; △, methylamine plus 10 or 20 mM sodium propionate; and ○, 1 or 10 mM monensin. Intracellular pH and K+ (50 mM) or nicotine (15 μM)-stimulated secretion were determined as shown in Fig. 1 and Fig. 4A. Secretion was plotted against intracellular pH at this time as a percentage of the control without treatment.

Fig. 6. Fluorescence recordings of acridine orange-loaded chromaffin cells. AO fluorescence is quenched due to uptake in acidic compartments. Recordings start when equilibration was completed. Where indicated, 10 mM NH4Cl (A) or 1 μM monensin (B) was added to the cells. An increase in fluorescence indicates alkalinization of the compartments. The results are semiquantitative and representative of two experiments each.

±28 nM, and in the presence of NH4Cl this (peak) value was 256 ± 19 nM (10 mM NH4Cl) or 343 nM (25 mM NH4Cl). With 20 mM sodium propionate, the peak value was somewhat lower, i.e. 203 ± 21 nM. The Ca2+ level at the plateau, or steady state, after the addition of secretagogue was 78 ± 5 nM. With NH4Cl (10 and 25 mM) this level was 70 and 97 nM, and with 20 mM sodium propionate it was 54 nM. It is clear from these data that NH4Cl did not inhibit the rise of the intracellular free Ca2+ concentration upon stimulation. On the contrary, NH4Cl had a tendency to increase the resting Ca2+ level and to enhance the increase of the intracellular Ca2+ concentration upon stimulation, especially at high concentrations. Sodium propionate, however, reduced the resting level and stimulated intracellular Ca2+ concentration and apparently accelerated the decay of the increased intracellular Ca2+ concentration upon stimulation.

Determination of Intracellular pH—In order to characterize the relation between the effects of NH4Cl, methylamine, sodium propionate, and monensin on secretion and on intracellular pH, we measured the intracellular pH (pHi) under various conditions leading to secretory inhibition using the fluorescent probe BCECF. The resting pH values varied between 6.75 and 7.05 and averaged 6.95 ± 0.07 (mean ± S.E., n = 5).

NH4Cl had a pronounced alkalinizing effect on the cytosolic pH (Fig. 4, A and B). The pH increase was rapid, i.e. it occurred within 20 s, and was partly reversed between 1 and 15 min after NH4Cl addition, probably due to the entry of NH4+ ions into the cell. The degree of alkalinization was dependent on the concentration of NH4Cl and was larger at higher concentrations (Fig. 4, A and B). The alkalinization by NH4Cl after a 1-min incubation was 0.09 pH units at 2.5 mM NH4Cl, 0.11 units at 5 mM, 0.17 units at 10 mM, and 0.23 pH units at 25 mM NH4Cl (averages of two to four experiments).

Methylamine (2.5–10 mM) caused a slower rise in pHi, which was not reversed (Fig. 4C). Instead, a steady state level of pH was attained after ~5 min of incubation which was dependent on the methylamine concentration. With 10 mM methylamine, pH increased by 0.08 units (average of two experiments).

Addition of sodium propionate (20 mM) caused a rapid decrease in intracellular pH of 0.28 pH units which was partly reversed to 0.07 pH units below the control in ~15 min (average of four experiments), probably by the action of a Na+-H+ exchanger (Fig. 4D). NH4Cl (10 mM) in combination with sodium propionate (10 mM) virtually had no effect on the cytosolic pH (not shown). NH4Cl (10 mM) plus sodium propionate (20 mM) changed the cell pH toward a slightly more acidic value as compared to the control (Fig. 4E). The acidification under these conditions was 0.06 pH units, which was restored to 0.03 pH units after 15 min (average of two experiments).

Monensin, which exchanges Na+ for protons, would be expected to alkalize the cytosol if its action is predominantly in the plasma membrane. Surprisingly, however, monensin (1 μM) acidified the cytosol to about the same extent as sodium propionate (0.26 pH units) within 1 min (Fig. 4F). The acidification was partially restored to ~0.08 pH units within 10 min, following a slightly different kinetic pattern than with sodium propionate.

Relation between the Effects on pH, and Catecholamine Secretion—Values obtained as described above for nicotine- or K+-stimulated catecholamine secretion were plotted as a function of intracellular pH (Fig. 5). This graph summarizes the results from experiments with various concentrations of NH4Cl and a preincubation time of 1 min, various preincubation times with 10 mM NH4Cl or methylamine, and a preincubation time of 1 min with 10 or 20 mM sodium propionate alone or in combination with 10 mM NH4Cl or methylamine. The relationship is not a simple titration curve since various secretory responses are found at one pH.

Acridine Orange Fluorescence Measurements—AO was accumulated in chromaffin cells, reflected by a decrease in its fluorescence due to quenching. Since the dye is weakly basic, it is concentrated to some extent in the cytosol, which is slightly acidic as compared to the extracellular medium, but mostly in the more acidic intracellular compartments, i.e. the chromaffin granules. We therefore considered the AO fluorescence quenching to represent mainly uptake into the granules. Addition of 10 mM NH4Cl under equilibrated conditions led to a decrease in fluorescence quenching, i.e. an efflux of AO out of the granules (Fig. 6A). This increased fluorescence is indicative of alkalinization of the granules. Monensin (1 μM) also alkalinized the granules, although at a slower rate and to a lesser extent than 10 mM NH4Cl (Fig. 6B). When NH4Cl (10 mM) was added 10 min after monensin (1 μM), additional alkalinization of the granules occurred (results not shown).
In this study we obtain evidence that the stimulated secretion from chromaffin cells is regulated by the pH of a cytosolic compartment, probably the chromaffin granule. Our basic findings are that the weak bases NH₄Cl and methyamine inhibit chromaffin cell secretion and that they alkalinate the cell interior, which is due to the rapid diffusion of the unprotonated base into the cell (21, 22). This alkalination will necessarily be most pronounced in acidic cell compartments such as the chromaffin granules.

Strong evidence implicating a role of this granule compartment in the secretory inhibition comes from the experiments with monensin. Monensin inhibits catecholamine secretion in a way which is apparently nonadditive to the inhibition by NH₄Cl, while it acidifies the cytosol. This suggests that NH₄Cl and monensin act at the same mechanism or site in the cell but not by affecting the cytosolic pH. In addition, the results of the experiments with acridine orange show that there is an acidic compartment in the cell which we believe to be the relatively abundant chromaffin granules and that the pH of this compartment is rapidly and clearly elevated upon the addition of NH₄Cl or monensin. Since these two agents both inhibit secretion and both increase the granule pH, while they alter the cytosolic pH in opposite ways, it seems likely that the increase of granule pH underlies the inhibition.

The fact that monensin, which is generally assumed to cause cytosolic alkalization due to coupled cellular Na⁺ influx and H⁺ efflux, causes cytoplasmic acidification is a surprising result. However, this assumption is based on the action of monensin in the cellular plasma membrane and does not take into account an incorporation of the ionophore in the chromaffin granule membrane, which would result in H⁺ efflux from the granule, since the Na⁺ and H⁺ gradients are both favorable (23) and thus lead to a countereffluxing cytosolic acidification.

Previous studies using other techniques than the AO fluorescence technique have shown that NH₄Cl and methyamine increase the pH of the chromaffin granule, which is normally somewhere between 5.3 and 5.8 (11, 12, 24). In permeabilized cells 30 mM NH₄Cl causes the pH of the granules to increase with 0.5–0.8 pH units (11), and in intact cells 20 mM NH₄Cl or methyamine increases the granule pH from 5.3 to 6.2 (12), as measured with the [14C]methyamine uptake technique. In isolated granules, NH₃ (10–40 mM) increases the pH of the granule by 0.8–1.5 pH units, from pH 5.8 to pH 6.6–7.3, as determined by 31P NMR (24).

Other evidence for a role of the granule pH in the inhibitory phenomenon is that the cytoplasmic alkalization by NH₄Cl is reversed in time, whereas the inhibition of secretion is persistent over the relevant time. In parallel, the results of the experiments with AO show that the alkalization of the granule by NH₄Cl is not reversed in time. Second, 20 mM sodium propionate completely reverses the cytosolic alkalization induced by 10 mM NH₄Cl but restores secretion only for a minor part. Theoretically, the latter treatment would, at a granule pH of 6.3, cause the influx into the granule of only 1.6 mM propionate. At a granule buffer capacity of 300 mM/pH unit/g of dry weight (25), i.e. 174 mM assuming a 58% water content (26), this would cause less than a 0.01 pH unit acidification. In the AO experiments, we found indeed that sodium propionate did not significantly acidify the granule or reverse the pH increase caused by NH₄Cl.

The arguments mentioned above are reflected in Fig. 5, in which we attempted to identify a correlation between cytoplasmic pH and secretion. The figure is clearly not a simple titration curve, and various secretory responses are found at one pH value throughout the relevant pH range. The figure also shows that the relation between pH and the secretory inhibition by NH₄Cl (various incubation times and concentrations) is different from that with methyamine (various incubation times, 10 mM). These deviations from an univocal relation also suggest that the inhibition of secretion by weak bases is, at least for a major part, due to alkalization of an intracellular compartment with a pH different from pHₙ, rather than to alkalization of the cytosol.

Therefore, we conclude that the secretory potency of chromaffin cells is related to the pH of the chromaffin granules. The quantitative relation between granule pH and secretion needs to be determined to further test this hypothesis, and at this point we cannot completely exclude that the cytosolic pH is an additional determining factor for secretion. For example, there is some restoration of NH₄Cl-inhibited secretion by sodium propionate, whereas it is not clear whether the granule pH can at all be affected by sodium propionate. Also, the pH we measure is an average over the cytosol, and local differences cannot be detected by our method. Therefore, an acidic area in the cytosol, e.g. close to the plasma membrane, could be an alternative site responsible for the inhibition. However, the results with monensin contradict this hypothesis since the ionophore acts at the membrane level, indicating that the inhibition is due to a pH change in a membrane-bound acidic compartment, and since monensin acidifies the cytosol whereas it inhibits secretion.

Inhibition of secretion by weak bases has been found in a number of cell types, such as β cells of the islets of Langerhans (27), neutrophils (28), egg cells (29), and plasma cells (30). On the other hand, acidification has been reported to increase secretion in turtle bladder (31) and in β cells (27). The inhibitory effect of weak bases has therefore been ascribed to the alkalization of either the cytosol or of an acidic subcellular compartment (28, 30). In chromaffin cells inhibition of catecholamine secretion by NH₄Cl has been reported recently and has been attributed to the NH₄Cl-induced rise in cytosolic pH (34).

The action of weak bases and acids has also been related to changes in the intracellular free Ca²⁺ concentration (31–33). From our experiments with Fura-2, however, we can exclude the concept that the inhibitory effect of NH₄Cl on secretion might be due to an effect of weak base on Ca²⁺, either through a decrease of the magnitude or a shortening of the duration of the Ca²⁺ transients. Therefore, we conclude that the inhibition of secretion due to the weak bases and monensin is the result of the inhibition of some step in the secretory process, which is subsequent to the stimulatory rise of Ca²⁺.

Historically, several attempts have been made to relate the pH or the ΔpH of the granules to the secretory events in chromaffin cells (35). In a study on electropermeabilized cells, Knight and Baker (10) concluded that Ca²⁺-stimulated secretion is not considerably affected by alteration of the pH over the range of 6.6–7.8 nor by agents that (partly) dissipate the pH gradient (NH₄Cl, 30 mM) or electrical potential gradient (carbonyl cyanide p-trifluoromethoxyphenyldrazone, 1 μM) across the granule membrane. In a subsequent paper on the involvement of the granule proton pump in secretion, the same authors found that NH₄Cl inhibits Ca²⁺-dependent secretion from electropermeabilized cells up to ~50% at 60 mM (pH 6.6) (11). However, the relationship between ΔpH across the granule membrane and secretion appeared to be very complex, and the authors concluded that the granular pH may play only a small modulating role in exocytosis (8). At a medium pH of 6.6, however, 60 mM NH₄Cl is equivalent to 0.0955 mM NH₃ and thus corresponds to ~11 mM NH₄Cl in

**DISCUSSION**

In this study we obtain evidence that the stimulated secretion from chromaffin cells is regulated by the pH of a cytosolic compartment, probably the chromaffin granule. Our basic findings are that the weak bases NH₄Cl and methyamine inhibit chromaffin cell secretion and that they alkalinate the cell interior, which is due to the rapid diffusion of the unprotonated base into the cell (21, 22). This alkalination will necessarily be most pronounced in acidic cell compartments such as the chromaffin granules.

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a medium of pH 7.35. At this concentration and medium pH, we found in our study that NH₄Cl causes the maximal inhibition of 50% in intact cells, and we conclude from this that the pH of the granule has an important role in secretion.

In an extensive study on the role of ΔΨM across the granule membrane, Holz et al. (12), using intact cultured chromaffin cells, investigated the effects of NH₄Cl, methylamine, nigericin, and other agents on secretion from chromaffin cells and measured the pH of the granule using [14C]methylamine and 31P NMR. In contrast to our results, their data showed that NH₄Cl and methylamine have no effect on stimulated secretion, and they concluded that ΔΨM does not play a role in exocytosis. However, as a stimulant they only used veratridine. Using this substance we did not find an inhibition by NH₄Cl and methylamine either but rather an enhancement of secretion. This result is surprising, but since the exact mechanism of the secretory stimulation of the Na⁺ channel agonist veratridine is not known and is only partially similar to the physiological pathway of stimulation, it is not possible to draw a conclusion from it. NH₄ ions might e.g. potentiate the depolarization caused by veratridine due to the permeability of the Na⁺ channels to NH₄ ions (36). In addition, a more recent study on intact chromaffin cells has shown that secretion is indeed inhibited by NH₄Cl when the cells are stimulated by K⁺-induced depolarization (34). In our study, we found secretion to be inhibited by both physiological stimulants nicotine and high K⁺.

In conclusion, we believe that the acidic pH in the chromaffin granule is essential for the exocytotic secretion of the granule content. The cytosolic pH may have a modulating role in the process. We emphasize that we are considering the pH of the granule or the cytosol per se and not the ΔpH or ΔΨM between the two compartments and that we cannot conclude anything about the role of H⁺ or ion transport or of the energy status of the granules in the secretory process. Alkalization of the chromaffin granule might lead to a premature termination of the secretory response, e.g. by inhibition of the fusion of (part of) the granules with the plasma membrane. Membrane fusion has indeed been shown to be pH-dependent in both biological and artificial systems (37, 38). This hypothesis would agree with the observed decrease in the rate constant of ATP secretion in the presence of NH₄Cl, found in this study, and could explain the fact that inhibition is maximally 50–60%. Preliminary electron micrograph data we obtained indeed show that in stimulated NH₄Cl-treated chromaffin cells more granules are located close to the plasma membrane in an apparently arrested state than in control stimulated cells. This hypothesis, however, needs to be investigated in detail.

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