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Otilonium: a potent blocker of neuronal nicotinic ACh receptors in bovine chromaffin cells

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1 Otilonium, a clinically useful spasmolytic, behaves as a potent blocker of neuronal nicotinic acetylcholine receptors (AChR) as well as a mild wide-spectrum Ca^{2+} channel blocker in bovine adrenal chromaffin cells.

2 $^{45}Ca^{2+}$ uptake into chromaffin cells stimulated with high K^+ (70 mM, 1 min) was blocked by otilonium with an IC_{50} of 7.6 μM. The drug inhibited the $^{45}Ca^{2+}$ uptake stimulated by the nicotinic AChR agonist, dimethylphenylpiperazinium (DMPP) with a 79 fold higher potency ($IC_{50}=0.096$ μM).

3 Whole-cell Ba^{2+} currents (I_{Ba}) through Ca^{2+} channels of voltage-clamped chromaffin cells were blocked by otilonium with an IC_{50} of 6.4 μM, very close to that of K^+-evoked $^{45}Ca^{2+}$ uptake. Blockade developed in 10–20 s, almost as a single step and was rapidly and almost fully reversible.

4 Whole-cell nicotinic AChR-mediated currents (250 ms pulses of 100 μM DMPP) applied at 30 s intervals were blocked by otilonium in a concentration-dependent manner, showing an IC_{50} of 0.36 μM. Blockade was induced in a step-wise manner. Wash out of otilonium allowed a slow recovery of the current, also in discrete steps.

5 In experiments with recordings in the same cells of whole-cell I_{DMPP}, Na^+ currents (I_{Na}) and Ca^{2+} currents (I_{Ca}), 1 μM otilonium blocked 87% I_{DMPP}, 7% I_{Na} and 13% I_{Ca}.

6 Otilonium inhibited the K^+-evoked catecholamine secretory response of superfused bovine chromaffin cells with an IC_{50} of 10 μM, very close to the IC_{50} for blockade of K^+-induced $^{45}Ca^{2+}$ uptake and I_{Ba}.

7 Otilonium inhibited the secretory responses induced by 10 s pulses of 50 μM DMPP with an IC_{50} of 7.4 nM. Hexamethonium blocked the DMPP-evoked responses with an IC_{50} of 29.8 μM, 4,000 fold higher than that of otilonium.

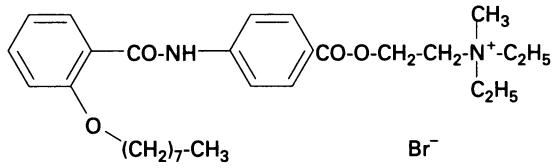
8 In conclusion, otilonium is a potent blocker of nicotinic AChR-mediated responses. The drugs also blocked various subtypes of neuronal voltage-dependent Ca^{2+} channels at a considerably lower potency. Na^+ channels were unaffected by otilonium. This extraordinary potency of otilonium in blocking nicotinic AChR, unrecognised until now, might account in part for its well known spasmolytic effects.

Keywords: Otilonium; nicotinic AChR; calcium channels; chromaffin cells; adrenal gland; catecholamine release

Introduction

Otilonium bromide is used clinically as a spasmolytic agent in intestinal disorders (Baldi *et al.*, 1991; Martindale, 1993). This drug is a quaternary ammonium derivative with a long aliphatic chain (Figure 1). It has been shown that otilonium blocks muscarinic receptors and reduces the contractions of rat colon induced by K^+ depolarization (Maggi *et al.*, 1983a). In addition, it has been proposed that it can interfere with Ca^{2+} mobilization, blocking both the cation entry and the release of Ca^{2+} from intracellular stores; however, no direct evidence is available to support this assumption (Maggi *et al.*, 1983b; Giachetti, 1991). Because of its poor gastrointestinal absorption, the drug has been claimed to be devoid of the side effects typical of the antimuscarinic agents used as spasmolytics (Maggi *et al.*, 1983a).

With this pharmacological antecedent, and because of the scarcity of compounds able to block neuronal voltage-dependent Ca^{2+} channels of the non-L subtypes (Olivera *et al.*, 1994), we thought it of interest to investigate the possibility that the compound had neuronal Ca^{2+} channel blocking actions. This was initially explored using $^{45}Ca^{2+}$ entry into bovine cultured chromaffin cells stimulated with the nicotinic agonist dimethylphenylpiperazinium (DMPP) or high K^+ concentrations, as a measure of Ca^{2+} channel activity (Gandía *et al.*, 1991). Initial experiments showed that otilonium was considerably more potent in blocking the nicotinic acetylcholine receptor (AChR)-mediated response than the K^+-evoked response. This suggested that otilonium, in addition to being a Ca^{2+} channel blocker, could behave as a potent antagonist of neuronal nicotinic AChR.



Otilonium bromide

Figure 1 Structural formula of otilonium bromide.

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The bovine adrenal medulla chromaffin cell in culture expresses neuronal nicotinic AChRs (Criado *et al.*, 1992; García-Guzmán *et al.*, 1995) and so, it is an excellent model to explore the above hypothesis. In this report, we present the results of the effects of otilonium on $^{45}Ca^{2+}$ entry, whole-cell Ba^{2+} currents (I_{Ba}), nicotinic AChR currents (I_{DMPP}), Na^+ currents (I_{Na}), Ca^{2+} currents (I_{Ca}) and catecholamine release in such a cell model. Otilonium behaved as a potent blocker of nicotinic AChR.

Methods

Isolation and culture of chromaffin cells

Bovine adrenal medullary chromaffin cells were isolated following standard methods (Livett, 1984) with some modifications (Moro *et al.*, 1990). Cells were suspended in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 5% foetal calf serum, 10 μM cytosine arabinoside, 10 μM fluorodeoxyuridine, 50 iu ml^{-1} penicillin and 50 $\mu g\ ml^{-1}$ streptomycin. Cells were plated at a density of 5×10^5 cells/well in 24-multiwell Costar plates for $^{45}Ca^{2+}$ uptake studies and were used 1–5 days after plating. For patch-clamp measurements cells were plated on circular glass coverslips. For secretion experiments, cells were plated in 60 mm diameter Petri dishes at a density of 5×10^5 cells ml^{-1}. Medium was replaced after 24 h and then after 2–3 days.

Measurements of $^{45}Ca^{2+}$ uptake

$^{45}Ca^{2+}$ uptake studies were carried out in cells after 2–3 days in culture. Before each experiment, cells were washed twice with 0.5 ml Krebs-HEPES solution of the following composition (mM): NaCl 140, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 1, glucose 11, HEPES 10, pH 7.2, at 37°C.

$^{45}Ca^{2+}$ uptake into chromaffin cells was studied by incubating the cells at 37°C with $^{45}CaCl_2$ at a final concentration of 5 $\mu Ci\ ml^{-1}$ in the presence of Krebs-HEPES (basal uptake), high K^+ solution (Krebs-HEPES containing 70 mM KCl with isoosmotic reduction of NaCl), or 100 μM dimethylphenylpiperazinium (DMPP) in Krebs-HEPES. This incubation was carried out during 1 min and at the end of this period; the test medium was rapidly aspirated and the uptake reaction was ended by adding 0.5 ml of a cold Ca^{2+}-free Krebs-HEPES containing 10 mM $LaCl_3$. Finally, cells were washed 5 times more with 0.5 ml of Ca^{2+}-free Krebs-HEPES containing 10 mM $LaCl_3$ and 2 mM EGTA, at 15 s intervals.

To measure radioactivity retained by chromaffin cells, the cells were scraped with a plastic pipette tip while 0.5 ml 10% trichloroacetic acid was added plus 3.5 ml scintillation fluid (Ready Micro, Beckman) and the samples were counted in a Packard beta counter. Results are expressed as % of $^{45}Ca^{2+}$ taken up by control cells.

Measurements of whole-cell currents

Membrane currents were recorded by the whole-cell configuration of the patch-clamp technique (Hamill *et al.*, 1981). Coverslips containing the cells were placed on an experimental chamber mounted on the stage of a Nikon Diaphot inverted microscope. The chamber was continuously perfused at room temperature (22–24°C) with a control Tyrode solution containing (in mM): NaCl 137, $MgCl_2$ 1, $CaCl_2$ 2, HEPES 10, glucose 10, pH 7.4 titrated with NaOH. For whole-cell I_{Ba} recording, 10 mM Ba^{2+} was used as the charge carrier and 5 μM tetrodotoxin (TTX) was added to the perfusion solution. Cells were dialysed with a standard intracellular solution containing (in mM): NaCl 10, CsCl 110, TEA.Cl 20, EGTA 14, HEPES 20, Mg.ATP 5, GTP 0.3, pH 7.2 titrated with CsOH.

For the recording in the same cells of DMPP-induced currents (I_{DMPP}), voltage-activated Na^+ currents (I_{Na}) and Ca^{2+} currents (I_{Ca}), brief (250 ms) pulses with an extracellular solution containing 100 μM DMPP were applied to a chromaffin cell voltage-clamped at −80 mV by means of a fast superfusion system (described below). Two seconds after each DMPP pulse, the cell was depolarized to +10 mV during 30 ms to elicit both I_{Na} and I_{Ca}. The extracellular solution contained 2 mM Ca^{2+} (instead of the 10 mM Ba^{2+} used to measure I_{Ba}) and no TTX. The intracellular solution had the same composition as that used to measure I_{Ba}.

Whole-cell recordings were made with fire-polished electrodes (resistance 2–5 MΩ) mounted on the headstage of a DAGAN 8900 (Dagan Corporation, Minneapolis, MN, U.S.A.) patch-clamp amplifier, allowing cancellation of capacitive transients and compensation of series resistance. A Labmaster data acquisition and analysis board and a 386-based microcomputer with pClamp software (Axon Instruments, Forster City, CA, U.S.A.) were used to acquire and analyse the data. Control and test solutions were changed with a multi-barreled concentration-clamp device, the common outlet of which was placed within 100 μm of the cell to be voltage-clamped. The flow rate (0.2–0.5 ml min^{-1}) was regulated by gravity. All experiments were performed at room temperature (22–24°C).

Cells were clamped at −80 mV holding potential (HP). Step depolarization to various test potentials (TP) from this HP lasted 25–50 ms and were applied at 10 s intervals to minimise the rundown of Ca^{2+} currents (Fenwick *et al.*, 1982). Cells with pronounced rundown were discarded.

Measurements of catecholamine release from superfused chromaffin cells

Bovine chromaffin cells (3×10^6) were trapped with glass wool in a microchamber (250 μl volume). Cells were continuously superfused at room temperature with a Krebs-HEPES solution of the following composition (in mM): NaCl 144, KCl 5.9, $MgCl_2$ 1.2, glucose 11 and HEPES 10 mM, at pH 7.4. The liquid flowing from the perfusion chamber reached an electrochemical detector model Metrohm 641VA under the amperometric mode, that monitored on-line the amount of catecholamines secreted (Borges *et al.*, 1986). The cells were stimulated to secrete by means of a Krebs-HEPES solution containing 1 mM Ca^{2+} and 50 μM DMPP, or high K^+ (70 mM, with isoosmotic reduction of NaCl), for 10 s at 5 min intervals. The secretory pulses with each secretagogue were applied by means of electrovalves, through an outlet placed 0.5 cm proximal to the microchamber. Known concentrations of adrenaline were used as external standards. Secretion was quantitated by measuring the peaks of the amperometric signals in nA.

Materials

Otilonium bromide was a kind gift of Dr Carganico (Menarini, Barcelona, Spain). Collagenase A was from Boehringer Mannheim (Madrid, Spain). DMEM, foetal calf serum, penicillin and streptomycin were purchased from GIBCO (Madrid, Spain). TTX, EGTA, 1,1-dimethyl-4-phenyl-piperazinium iodide (DMPP), nicotine, acetylcholine choride and hexamethonium chloride were purchased from Sigma Chemical (UK). ^{45}Ca (specific activity 10–40 mCi mg^{-1} calcium) was purchased from Amersham and scintillation fluid Ready Micro from Beckman. Salts used in the preparation of buffers were reagent grade and were obtained from Merck (Madrid, Spain).

Statistical analysis

Results are expressed as means ± s.e.mean. Concentration-response curves for the effects of otilonium on $^{45}Ca^{2+}$ uptake, nicotinic AChR currents and catecholamine release were analysed by a Graph PAD software programme. In all cases, the logarithmic transformation of the sigmoid concentration-response curves fitted straight lines with r^2 between 0.992 and 0.998.

Results

Effect of otilonium on $^{45}Ca^{2+}$-uptake into bovine chromaffin cells

First we wished to compare the effects of otilonium (see formula in Figure 1) on $^{45}Ca^{2+}$ entry into bovine chromaffin cells depolarized with 70 mM K^+ or the nicotinic AChR agonist, DMPP. Cells were exposed to solutions containing 70 mM K^+ or 100 μM DMPP for 60 s. These concentrations of DMPP and K^+ are known to stimulate maximally the $^{45}Ca^{2+}$ uptake by these cells (Gandía *et al.*, 1991). $^{45}Ca^{2+}$ uptake was studied in the absence (control) and the presence of increasing concentrations of the drug. Before stimulation, cells were preincubated with each concentration of otilonium for 10 min. Otilonium was also present during the depolarizing period.

K^+ stimulation induced $^{45}Ca^{2+}$ uptake (in the presence of 1 mM $^{40}Ca^{2+}$) of 6,065 ± 660 c.p.m. (data from 8 individual wells from 3 different batches of cells). In the same conditions, 100 μM DMPP induced $^{45}Ca^{2+}$ uptake of 4,494 ± 306 c.p.m. (data from 8 individual wells from 3 different batches of cells). In basal conditions (1 min incubation in Krebs-HEPES solution contining 5 μCi ml^{-1} $^{45}Ca^{2+}$ plus 1 mM $^{40}Ca^{2+}$), cells retained 877 ± 69 c.p.m. (data from 14 individual wells from 5 different batches of cells). Thus, the mean ratio evoked/basal Ca^{2+} uptake was 6.9 for 70 mM K^+ and 5.1 for DMPP.

Otilonium inhibited K^+- and DMPP-evoked Ca^{2+} uptake into chromaffin cells in a concentration-dependent manner (Figure 2). The concentration-response curves were very steep (Hill coefficients around 3). The IC_{50}s to inhibit the responses were 7.6 and 0.096 μM, respectively for 70 mM K^+ and DMPP. Thus, otiolonium was 79 fold more potent in inhibiting the nicotinic AChR-mediated response than the response to direct cell depolarisation with 70 mM K^+.

Blockade by otilonium of I_{Ba}

Inhibition by otilonium of $^{45}Ca^{2+}$ uptake induced by 70 mM K^+ was probably due to blockade of Ca^{2+} channels. Therefore, a parallel blockade of whole-cell I_{Ba} was expected. The time-course of I_{Ba} elicited by 50 ms depolarizing pulses, given at 10 s intervals to a voltage-clamped chromaffin cell (−80 mV holding potential) is shown in Figure 3. I_{Ba} initially stabilized at about 1,400 pA; then, when superfusion of the cell with 10 μM otilonium started, I_{Ba} was reduced to 300 pA. The blockade developed fully in about 20 s and remained during exposure to the drug (about 90 s). Washout of otilonium induced a fast recovery of I_{Ba} (90% recovery in 20 s). Reapplication of the compound two times more produced the same blockade and recovery. The inset in Figure 3 shows the original traces of I_{Ba} obtained from the points a, b and c in the time course curve. Current-voltage curves (from −60 to +60 mV) recorded in the absence and the presence of 10 μM otilonium showed no apparent shifts (not shown).

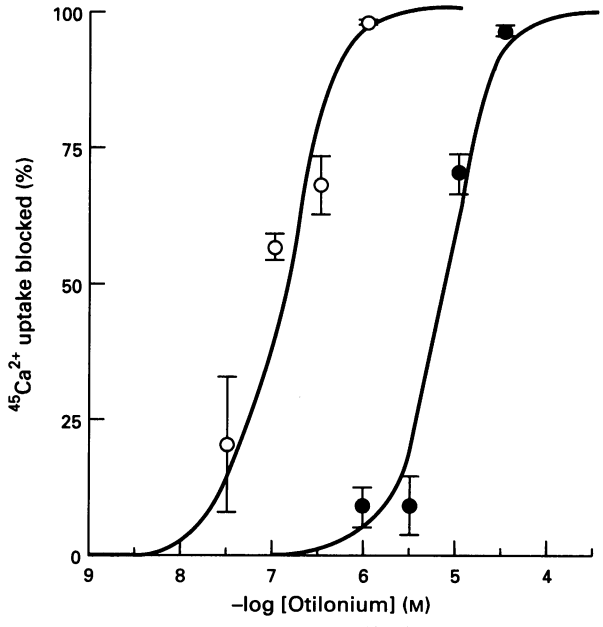

Figure 2 Effect of otilonium on $^{45}Ca^{2+}$ uptake into bovine chromaffin cells stimulated with 70 mM K^+ (●) or 100 μM DMPP (○) for 60 s. Before stimulation, cells were preincubated with each concentration of otilonium for 10 min. In each experiment, $^{45}Ca^{2+}$ uptake (ordinate scales) was normalized to $^{45}Ca^{2+}$ taken up by cells in the absence of drug. Data are means ± s.e.mean of 8 wells from 3 different batches of cells.

Figure 4 analyses the concentration-dependence of otilonium for blockade of I_{Ba}. The threshold concentrations were between 1 and 3 μM and maximum inhibition (over 90%) was achieved at 30 μM (Figure 4a). Figure 4(b) shows averaged results from different cells and cultures. They were obtained from cells subjected to the protocol shown in (a), though many results were also taken from cells treated with single otilonium concentrations. This was because repeated applications of otilonium to the same cell led to an incomplete recovery of I_{Ba}, which underwent a progressive decline. Figure 4b shows a concentration-response curve for the blocking effects of otilonium on I_{Ba}. As for $^{45}Ca^{2+}$ uptake (Figure 2), the curve was steep (Hill slope coefficient around 1.5), showing a very similar IC_{50} (6.4 μM).

Effects of otilonium on I_{DMPP}

In view of the higher potency of otilonium for blockade of $^{45}Ca^{2+}$ uptake induced by DMPP, its effects on I_{DMPP} were initially explored at lower concentrations. Figure 5a shows original traces of I_{DMPP} triggered by the application at 30 s intervals of 250 ms pulses of DMPP (100 μM) to a voltage-clamped chromaffin cell (holding potential −80 mV). With such brief applications of DMPP, desensitization of the nicotinic AChR was low and reproducible currents could be elicited for at least a 15–20 min period. In 204 pulses of DMPP given to 41 cells from different cultures, the average amplitude of I_{DMPP} was 2,217 ± 50 pA.

Figure 5b shows the effect of otilonium (1 μM) on repetitively activated I_{DMPP}. Otilonium reduced I_{DMPP} in successive steps, to a final size of only 10% of the initial current. Upon washout of the drug, I_{DMPP} recovered, again in discrete steps. This step-wise manner of blocking I_{DMPP} contrasts with the blockade of I_{Ba}, which was faster and developed in about 10–20 s (compare Figures 3 and 5b). Figure 5c shows the concentration-response curve for otilonium blockade of I_{DMPP}. At 0.1 μM, I_{DMPP} was blocked 20%; 1 μM caused almost full inhibition. Thus, the inhibitory curve was quite steep (Hill coefficient of 1.6). The IC_{50} for otilonium to block I_{DMPP} was 0.36 μM, about 18 fold lower than its IC_{50} to block I_{Ba}.

To study further the effects of otilonium on the kinetics of I_{DMPP}, nicotinic AChR currents were elicited with longer pulses of DMPP (1 s) at a lower concentration (10 μM), to see better the rate of desensitisation of the current. Figure 6a shows the profiles of I_{DMPP} in an expanded time scale. The control inward current activated with a τ of 20 ms, reached a peak of about 2,000 pA and then inactivated. Otilonium (1 μM) decreased the peak I_{DMPP} in discrete steps and in a time-dependent manner. However, otilonium did not accelerate the rate of desensitization of I_{DMPP}. Figure 6b shows the time-course of the blocking effects of otilonium on a slower time scale. The step-wise blockade of I_{DMPP} suggests a use-dependent block. The experiment of Figure 6c corroborates this. I_{DMPP} currents were initially elicited at 30 s intervals. When currents stabilized, otilonium was superfused for 3 min in the absence of DMPP stimulation (Figure 6c). Then, DMPP pulses were applied again at 30 s intervals. After this 'silent' period, otilonium

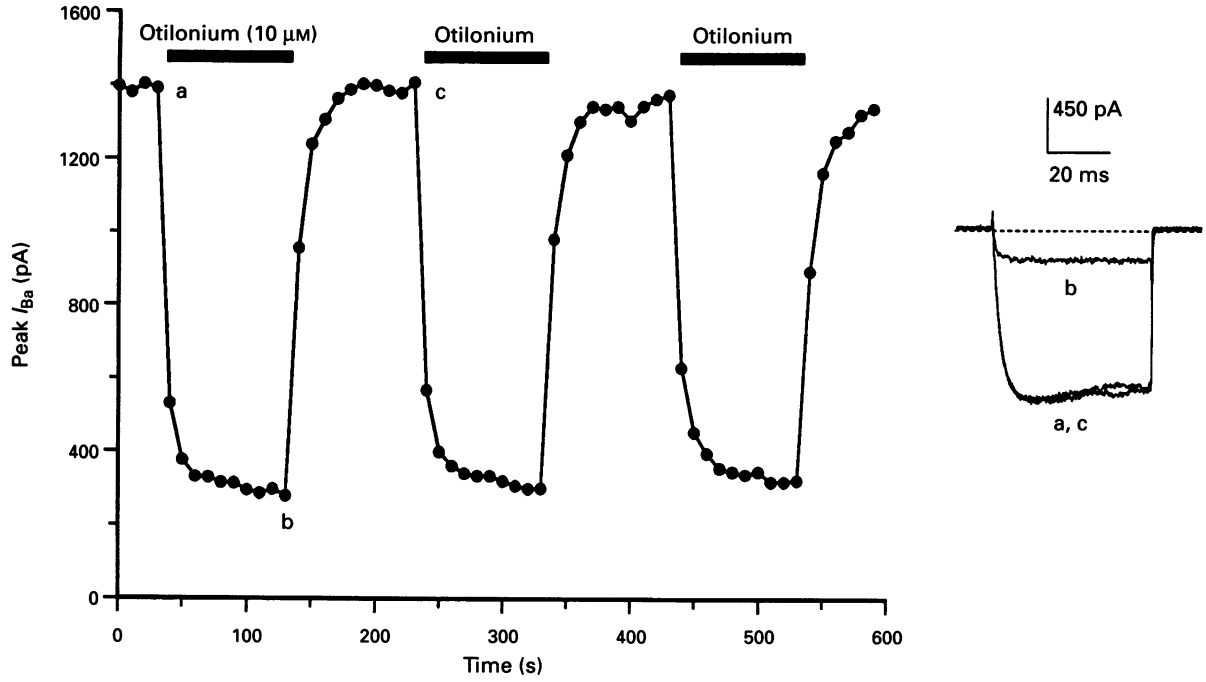

Figure 3 Reversible blockade by otilonium of I_{Ba}. Test depolarizing pulses to 0 mV, from a holding potential of −80 mV were applied at 10 s intervals to a voltage-clamped chromaffin cell. The figure shows the time course of I_{Ba}. Otilonium (10 μM) was applied as shown on the horizontal bars at the top of the figure. Inset, original traces obtained before, during and after washing out otilonium, taken at the points identified with the letters a, b, and c on the time course curve.

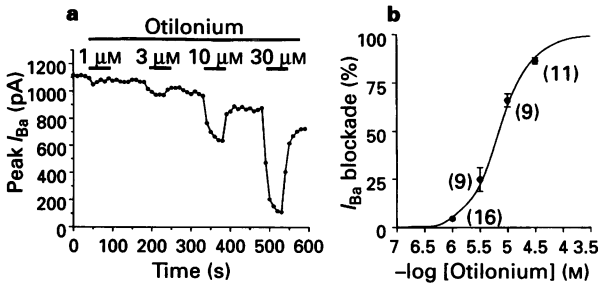

Figure 4 Effect of increasing concentrations of otilonium on I_{Ba}. (a) Shows the effect of increasing concentrations of otilonium on I_{Ba}. After breaking into a chromaffin cell with the patch pipette (holding potential −80 mV), depolarizing pulses to 0 mV were applied at 10 s intervals. Ba^{2+} (10 mM) was used as the charge carrier. Increasing concentrations of otilonium (1–30 μM) were applied to the cell as indicated by the top horizontal bars. (b) Shows the concentration-response curve for the inhibitory effects of otilonium on I_{Ba}. Data were obtained from cells treated with single concentrations of otilonium. They were normalized as % of the I_{Ba} amplitude obtained immediately before addition of each drug concentration. They are means ± s.e.mean of the number of cells shown in parenthesis.

blocked I_{DMPP} 38 ± 5.7% (n = 5 cells). This contrasts with the much greater blockade achieved after 3 min without the silent period (82 ± 3.3%; n = 6).

Comparative effects of otilonium on I_{DMPP}, I_{Na} *and* I_{Ca}

The question of whether otilonium blocks selectively the nicotinic AChR ionophore or other ion channels as well, was explored by use of the following protocol (see Figure 7). A voltage-clamped chromaffin cell was stimulated first with a pulse of DMPP (100 μM for 250 ms) and 2 s later with a 30 ms depolarizing test pulse to + 10 mV. This pair of chemical and electrical stimuli were repeated at 30 s intervals. This stimulation pattern allowed the recording in the same cell of I_{DMPP}, I_{Na} and I_{Ca} (see Methods for the ionic composition of the extracellular and intracellular solutions). On repeated stimulation, the three ionic currents remained stable.

Averaged currents from 26 cells amounted to 2,007 ± 130 pA for I_{DMPP}, 844 ± 70 pA for I_{Na} and 384 ± 29 pA for I_{Ca}. Typical traces from one of those 10 cells are shown in Figure 7b. Trace (i) shows the I_{DMPP} recorded in control conditions, trace (ii) shows the current recorded 2 min after superfusion with 1 μM otilonium, and trace (iii) was obtained 6 min thereafter. Blockade of I_{DMPP} developed in a clear stepwise manner to end with the suppression of the current after 5–8 DMPP pulses. The peak I_{Na} and I_{Ca} declined about 15 and 16%, respectively, during otilonium application. I_{Na} was blocked 7.3 ± 1.9% (n = 10 cells) by 1 μM otilonium and 18 ± 7% (n = 5 cells) by 3 μM. On the other hand, I_{Ca} was depressed 12.8 ± 2.5% (n = 11 cells) by 1 μM and 21.7 ± 5.2% (n = 5 cells) by 3 μM of the drug.

Effects of otilonium on K^+*-evoked catecholamine release*

On-line catecholamine release following direct depolarization was studied by applying 10 s pulses at 5 min intervals, of a K^+-enriched solution (70 mM K^+, 2 mM Ca^{2+}) to chromaffin cells superfused with a Krebs-HEPES solution. This stimulation pattern produced reproducible secretion peaks for at least a 40 min period (see Figure 8a). The average magnitude of these peaks was 230 ± 28 nA (n = 8 pulses). At the perfusion rate used in these experiments (3 ml min^{-1}) this current was equivalent to 833 ng of adrenaline per K^+ pulse.

Otilonium (10 μM) was given during a 15 min period and three successive K^+ challenges were applied in its presence. Secretion was depressed by 18%, 24% and 30% respectively for the first, second and third challenge (Figure 8b). After washout of the drug, recovery of the secretory response in three subsequent K^+ challenges was gradual but incomplete. Figure 8c shows a concentration-response curve for the blocking effects of otilonium of K^+-induced secretion. Its IC_{50} was 11.5 μM, very close to the IC_{50} for blocking K^+-evoked $^{45}Ca^{2+}$ entry (Figure 2) and I_{Ba} (Figure 4b).

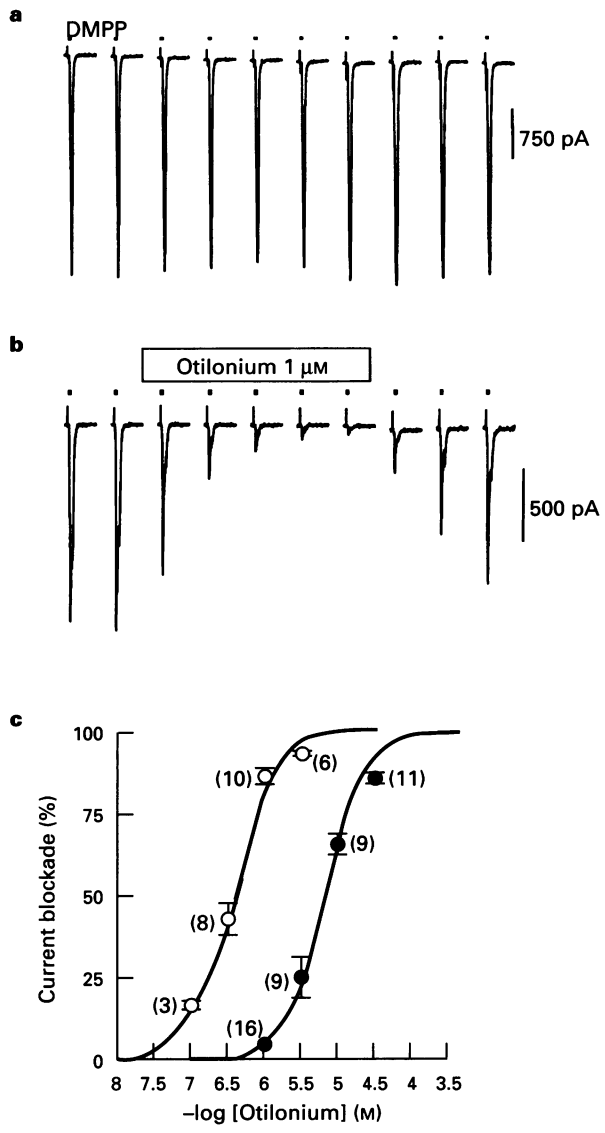

Figure 5 Effect of otilonium on nicotinic receptor currents. Currents were elicited by pulses of DMPP (100 μM, 250 ms) applied at 30 s intervals to voltage-clamped chromaffin cells (holding potential −80 mV). (a) Shows original current traces evoked by repetitive pulses; this stimulation pattern gave reproducible currents of similar size. In (b) the time-course of the blocking effect of otilonium (1 μM) on I_{DMPP}, is shown. In (c), the concentration-response curve for the blocking effect of otilonium on I_{DMPP} (○) is plotted. For comparative purposes, the concentration-response curve on the effect of otilonium on I_{Ba} (●) has been redrawn from Figure 4b. Data are means ± s.e.mean of the number of cells shown in parentheses near each symbol.

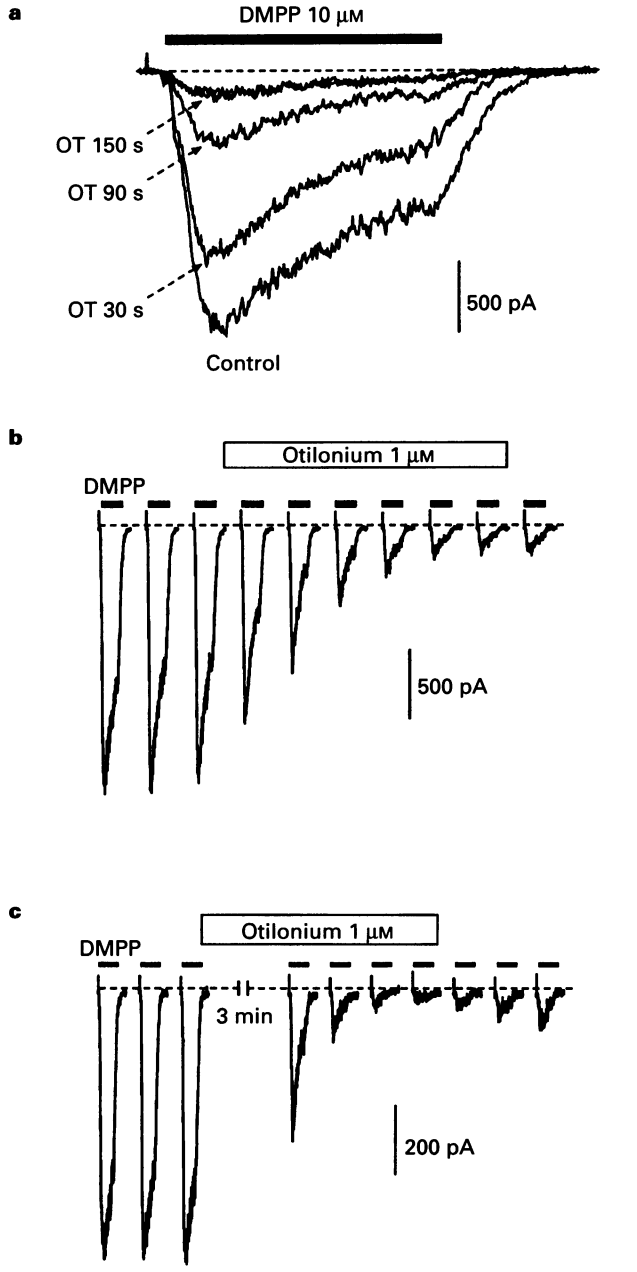

Figure 6 Effects of otilonium (1 μM) on nicotinic AChR currents elicited with a low concentration of DMPP (10 μM) for a longer pulse duration (1 s). In (a), a voltage-clamped chromaffin cell (holding potential −80 mV) was stimulated with DMPP at 30 s intervals. Otilonium (OT) was applied at the times shown in the traces. In (b), the time-course of the blockade induced by otilonium, with uninterrupted application of DMPP pulses at 30 s intervals, is shown. In (c), three initial pulses of DMPP were applied at 30 s intervals. Then otilonium was applied while DMPP stimulation was interrupted for 3 min. After this 'silent' period, DMPP pulses were resumed at 30 s intervals.

Effects of otilonium on DMPP-evoked catecholamine release

Pulses of 50 μM DMPP and 10 s in length, applied at 5 min intervals produced an initial secretory response of 133 ± 15 nA (n = 20 pulses). Such a response declined in successive pulses to reach about 70% of the initial signal at the 10th pulse. The effects of various otilonium concentrations on secretion were studied by intercalating a single concentration between the DMPP pulses, in every group of cells studied. Usually, two to three initial 10 s pulses of 50 μM DMPP were applied in the absence of the drug. Figure 9a shows the pattern of secretory responses elicited by DMPP in control cells. Figure 9b shows the effects of 1 μM otilonium on such secretory signals. After 5 min of superfusion with the drug the secretory response was abolished. Secretion was still suppressed during the two further DMPP pulses given in the presence of otilonium. Then, during the washout period, the DMPP response recovered partially and very slowly. The blocking effects of 100 μM hexamethonoium, a typical ganglionic nicotinic AChR blocker, are shown in Figure 9c. After 15 min, blockade amounted to 60% and recovery from blockade was practically complete after 5 min wash out of hexamethonium. Figure 9d shows concentration-response curves for the blocking effects of otilonium compared with hexamethonium. The IC_{50} for otilonium was 7.4 nM and for hexamethonium 29.8 μM.

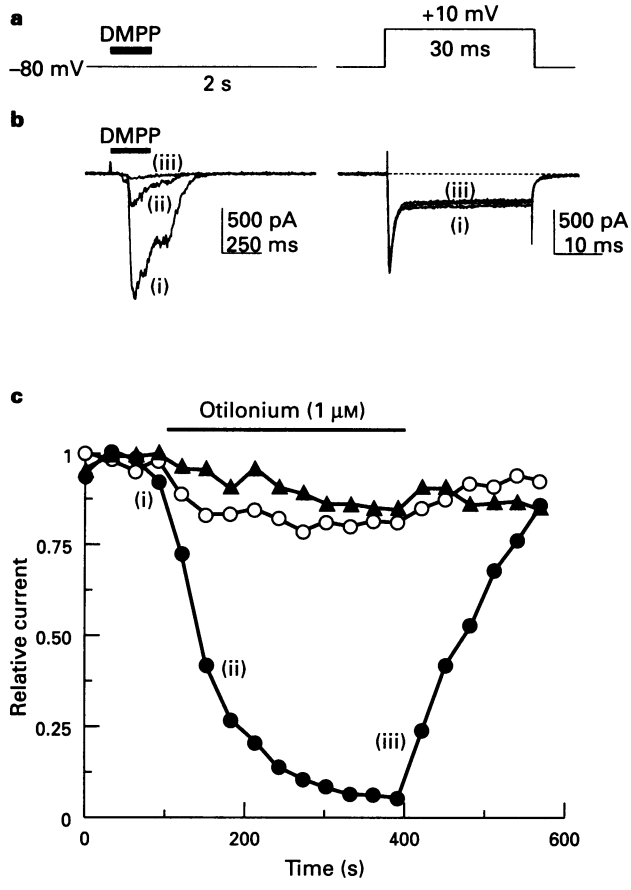

Figure 7 Different effects of otilonium on nicotinic AChR currents (I_{DMPP}), Na^+ currents (I_{Na}) and Ca^{2+} currents (I_{Ca}). The three ion currents were simultaneously measured in a voltage-clamped chromaffin cell (holding potential −80 mV) using the protocol shown in (a). A pulse of DMPP (100 μM, 250 ms) was first given to induce I_{DMPP} (first traces in panel (b)). After 2 s, a depolarizing 30 ms pulse to +10 mV was applied, to measure I_{Na} and I_{Ca} (peak and plateau in the trace to the right in panel b). Panel (c) shows the time course of the three currents elicited at 30 s intervals in the absence and the presence (top horizontal bar) of otilonium (1 μM): (●) I_{DMPP}; (○) I_{Na}; (▲) I_{Ca}. The original current traces in (b) were taken at the points identified by (i), (ii), (iii) in (c).

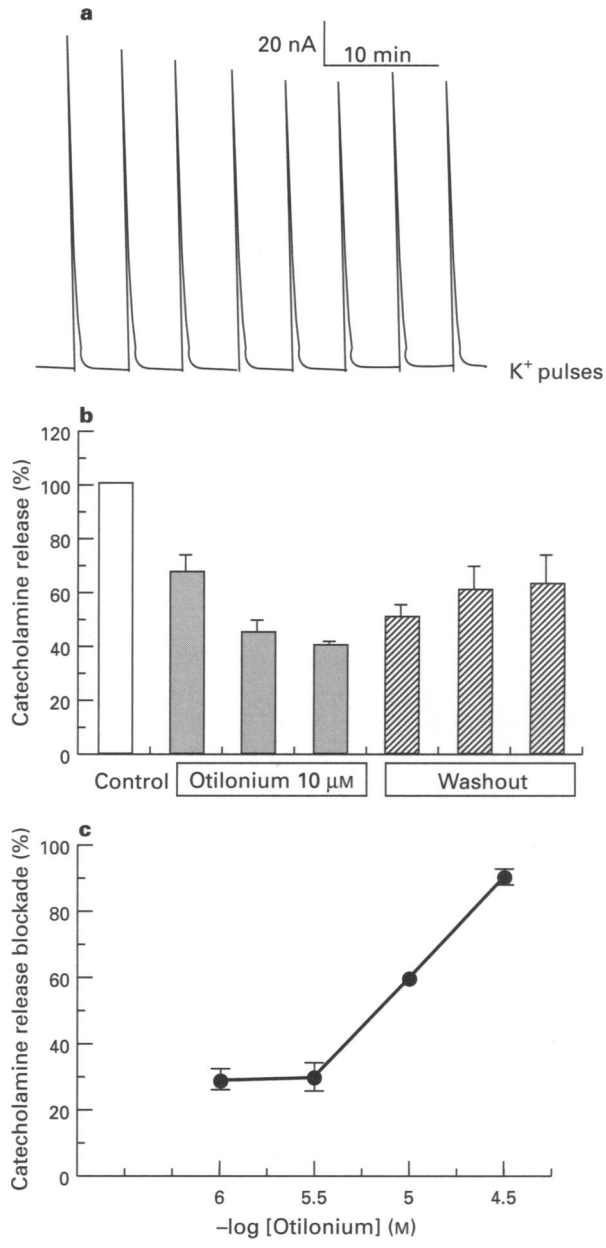

Figure 8 Effects of otilonium on K^+-evoked catecholamine release from superfused chromaffin cells. In (a), cells were challenged with a Krebs-HEPES solution enriched in K^+ (70 mM) for 10 s, at 5 min intervals. The secretory responses are expressed in nA (see calibration bars). In (b), the effect of otilonium (horizontal bar) on secretion is shown. Data are means ± s.e.mean of 4 experiments. In (c), a concentration-response curve for the inhibitory effects of otilonium on secretion is shown. Data are means ± s.e.mean.

Discussion

This study shows that otilonium behaves as a powerful blocker of nicotinic AChR-mediated responses in bovine adrenal chromaffin cells. Though the K^+-evoked responses were also blocked, its potency was far weaker. For instance, IC_{50} to block K^+-induced $^{45}Ca^{2+}$ entry, I_{Ba} and K^+-evoked catecholamine release were respectively, 8.4 μM, 6.4 μM and 10 μM. In contrast, IC_{50}s to block DMPP-induced $^{45}Ca^{2+}$ entry, I_{DMPP} and DMPP-evoked catecholamine release were, respectively, 0.1 μM (79 fold lower), 0.36 μM (18 fold lower) and 0.01 μM (1000 fold lower). In addition, clear differences were seen in the kinetics of block and unblock by otilonium of I_{Ba} and I_{DMPP}. No effects of otilonium on I_{Na} were observed.

The concentration-dependent blockade by otilonium of $^{45}Ca^{2+}$ uptake and catecholamine release triggered by K^+ depolarization, as well as by the inhibition of whole-cell I_{Ba} suggest that the drug has Ca^{2+} channel blocking properties. As in neurones (Olivera *et al.*, 1994), multiple types of voltage-dependent Ca^{2+} channels have been discovered and characterized recently in bovine adrenal medulla chromaffin cells (Gandía *et al.*, 1994). Because otilonium inhibited almost completely the whole-cell I_{Ba} at the higher concentration used (10 μM), it seems clear that at these concentrations otilonium does not distinguish between Ca^{2+} channel subtypes. In this sense, this compound shares the wide spectrum Ca^{2+} channel blockade of drugs such as R56865 (Garcez-do-Carmo *et al.*, 1993), dotarizine and flunarizine (Villarroya *et al.*, 1995) In contrast to the highly lipophilic compounds R56865, dotarizine and flunarizine, otilonium is a hydrophilic compound containing a permanently charged quaternary ammonium in its molecule. This is an important difference with the lipophilic Ca^{2+} channel blockers which is reflected in the rate of block and unblock of the current. R56865, dotarizine and flunarizine induced a slowly developing blockade of I_{Ba} in bovine chromaffin cells. Although such blockade was readily reversible in the case of R56865 (Garcez-do-Carmo *et al.*, 1993), it was more slowly reversible in the case of flunarizine (Villarroya *et al.*, 1995). Blockade of I_{Ba} by otilonium was fully established

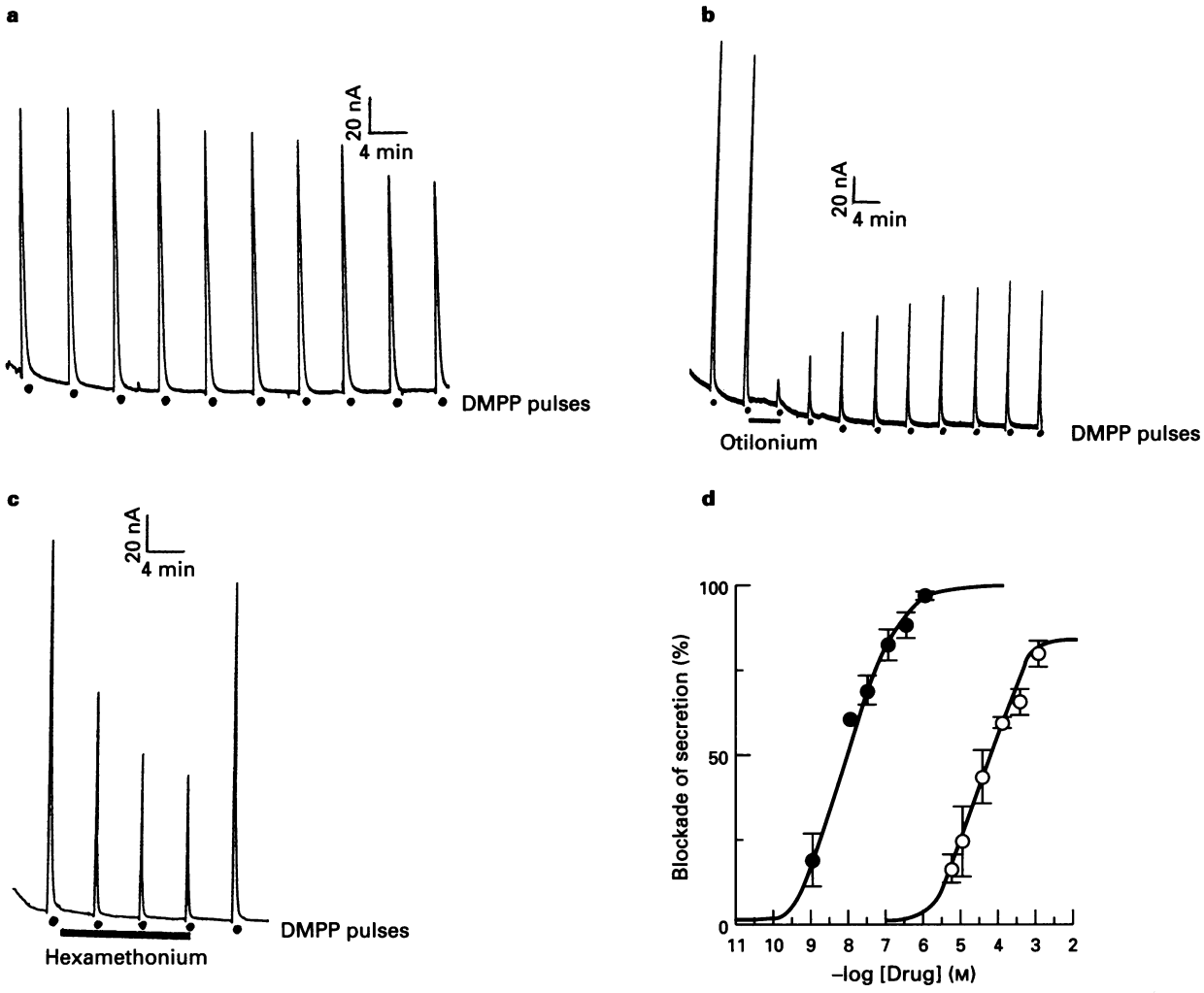

Figure 9 Inhibition by otilonium and hexamethonium of nicotinic AChR-mediated catecholamine release, and recovery from blockade. In the experiment shown in (a), cells were challenged with DMPP pulses (50 μM at 5 min intervals). Panel (b) shows the effects of otilonium (1 μM) on DMPP secretory responses, and their partial recovery after washout of the drug. Panel (c) shows the inhibitory effects of hexamethonium (100 μM) on DMPP secretory responses and its fast recovery after wash out. (d) Shows the concentration-response curves for the blocking effects of otilonium (●) and hexamethonium (○). Data are means ± s.e.mean of 4–6 experiments for each drug concentration. They were normalized to the initial secretory responses to DMPP and were not corrected for the gradual decline seen in subsequent DMPP pulses (a).

after 10–20 s, especially at a concentration of 10 μM and was quickly reversible after washing out the drug. This can be related to its polarity and high water solubility.

More interesting were the effects of otilonium on nicotinic AChR-mediated responses. In addition to its higher potency (18 fold more potent than its actions on Ca^{2+} channels), the blockade of nicotinic AChR exhibited interesting features. For instance, the mechanism of blockade differed considerably from that of the classical ganglionic blocking agent, hexamethonium. The blockade of the nicotinic AChR by hexamethonium seems to involve both competition with acetylcholine and block at the nicotinic AChR-associated cationic channel pore (Rang, 1982; Gurney & Rang, 1984). The effects of otilonium on I_{DMPP} are not compatible with a mechanism involving an acceleration of the closing of open nicotinic AChR channels. Rather, the step-wise blockade of I_{DMPP} (Figures 5 and 6), and its poorer blockade in unstimulated cells (Figure 6c) support a use-dependent inhibition by otilonium of the nicotinic AChR ion channel. This behaviour sharply contrasts with the rapid blockade and rapid restoration of I_{Ba} after introduction and removal of otilonium (Figure 3). So, in the case of Ca^{2+} channels, there seems to be no use-dependence of the blocking effects of otilonium, since it developed fully immediately after its introduction. But in the case of nicotinic AChR channels, the use-dependence implies a stable attachment of otilonium to the channel pore, which might explain the slow step-wise recovery of I_{DMPP} upon wash out. This behaviour might be conditioned by the molecular structure of otilonium (Figure 1) which includes a quaternary ammonium which might easily recognise the nicotinic AChR pore, and a long aliphatic chain that might favour its insertion into the lipids surrounding the nicotinic AChR and its slow wash out. In contrast to otilonium, the higher polarity of hexamethonium (two quaternary ammoniums and no lipophillic groups) may explain its rapid reversibility.

As stated in the Introduction, otilonium is widely used to treat disorders of intestinal motility, including irritable colon syndrome. Various recent clinical trials have demonstrated its efficacy in this condition (Baldi *et al.*, 1991; Giachetti, 1991). On the other hand, pinaverium, a blocker of L-type Ca^{2+} channels in intestinal smooth muscle (Feron *et al.*, 1992; Bobo *et al.*, 1994) has comparative efficacy to otilonium in this condition. As for pinaverium, the Ca^{2+} channel blocking properties shown here for otilonium might contribute to the reduction of the intestinal tonus and motility. However, blockade of nicotinic AChR at parasympathetic ganglia of the Auerbach plexus might also contribute to the spasmolytic effects of otilonium. Its poor gastrointestinal absorbtion, due to its high polarity, will preclude any systemic ganglionic blocking action.

In conclusion, otilonium exhibits potent blocking effects of neuronal nicotinic AChR and milder inhibitory actions on voltage-dependent Ca^{2+} channels. Otilonium did not affect the Na^+ channels. These actions might explain its well established spasmolytic effects. This study might also open new pathways and strategies for the search for drugs to treat gastrointestinal disorders: like otilonium, compounds that produce combined inhibition of these subtypes of channels may be useful for this treatment. In addition, the results presented here may provide a clue to the development of novel neuronal nicotinic AChR agonists and antagonists.

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