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Blocking effects of otilonium on Ca^{2+} channels and secretion in rat chromaffin cells

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Abstract

We describe here the effects of otilonium bromide (an anticholinergic agent widely used as an intestinal spasmolytic) on whole-cell currents through Ca^{2+} channels (I_{Ba}) and catecholamine secretion in rat adrenal glands and isolated rat chromaffin cells. Otilonium blocked the peak I_{Ba} current in voltage-clamped chromaffin cells in a concentration-dependent manner; the IC_{50} to block I_{Ba} was 4.7 μM. Blockade was not accompanied by a significant shift in the *I-V* relationship for I_{Ba}, suggesting that such blockade was not affecting a specific subtype of Ca^{2+} channel. When given intracellularly through the patch pipette, otilonium (10 μM) did not block I_{Ba}. However, its external application to the same cell (10 μM) reversibly reduced I_{Ba} by 70%. Otilonium caused a concentration-dependent blockade of catecholamine release from perfused rat adrenal glands intermittently stimulated with methacholine, high K^+ or histamine. The IC_{50} to block secretion after a 5 min incubation with otilonium was 0.02, 0.7 and 3 μM, respectively, for methacholine, K^+ and histamine. The blocking effects of otilonium were fully reversible at concentrations below 10 μM. The Ca^{2+} channel agonist Bay K 8644 (methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate) partially antagonized the effects of otilonium on K^+-evoked secretion and accelerated the time course of recovery from inhibition. The results are compatible with the idea that otilonium blocks Ca^{2+} entry into chromaffin cells by blocking voltage-dependent Ca^{2+} channels. This would lead to a limitation in the rise in cytosolic Ca^{2+} at secretory sites and to inhibition of catecholamine release in response to stimulation of chromaffin cells.

Keywords: Otilonium; Chromaffin cell; Ca^{2+} channel; Adrenal gland; Catecholamine release

1. Introduction

Otilonium is a quaternary ammonium derivative with a long aliphatic chain (Fig. 1). For years, otilonium bromide has been widely used as a spasmolytic agent for intestinal disorders (Baldi et al., 1991). 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 otilonium 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).

With this pharmacological profile, we thought that otilonium could interfere with Ca^{2+} homeostasis and secretion in chromaffin cells. We selected the rat adrenal gland as a model to test this hypothesis because it possesses different subtypes of voltage-dependent Ca^{2+} channels in the plasmalemma (Gandía et al., 1995) and the release of catecholamines from its medullary cells is controlled by Ca^{2+} entry through L and non-L subtypes of voltage-dependent Ca^{2+} channels (López et al., 1992). Therefore, this is a suitable model to explore whether otilonium affects both Ca^{2+} channels, Ca^{2+} entry and secretion. For this purpose, the effects of the drug on whole-cell currents through Ca^{2+} channels and cate-

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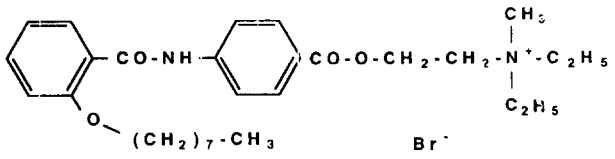

Fig. 1. Chemical structure of otilonium bromide.

cholamine release were studied in such cells. The results of this study are presented here.

2. Materials and methods

2.1. Isolation and culture of rat chromaffin cells

Rats of both sexes were killed by cervical dislocation. The abdomen was opened and both adrenals were exposed. Adrenal medullae were extruded through a small cut made on the cortex. Medullae from five rats were placed into a sterile Ca^{2+}- and Mg^{2+}-free Locke solution containing (in mM): NaCl 144, KCl 5.6, glucose 11, Hepes 10. The pH was adjusted to 7.4 with NaOH.

Tissues were trimmed and placed in 2 ml Locke solution containing collagenase (0.15%) and bovine serum albumin (0.3%) for 30 min at 37°C. Every 5 min the pieces of adrenal tissue were disrupted by passing them several times through a plastic Pasteur pipette tip. The material was filtered through a 200 μm nylon mesh, centrifuged at $120 \times g$ and washed twice with fresh Locke solution. The pellet was resuspended in 10 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum and containing 50 IU ml^{-1} penicillin and 50 μg ml^{-1} streptomycin, and plated on circular glass coverslips. Cells were used within 1–3 days of culture.

2.2. Measurements of Ba^{2+} currents

Membrane currents were recorded using the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981). Coverslips with the cells were placed on an experimental chamber mounted on the stage of a Nikon Diaphot inverted microscope. The chamber was continuously perfused 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 current recording, 10 mM Ba^{2+} was used as the charge carrier and 5 μM tetrodotoxin was added to the perfusion solution. Cells were dialysed with a standard intracellular solution containing (in mM): NaCl 10, CsCl 110, tetraethylammonium · Cl 20, EGTA 14, Hepes 20, Mg · ATP 5, GTP 0.3, pH 7.2 titrated with CsOH.

Whole-cell recordings were made with fire-polished borosilicate glass (Kimax 51) electrodes (resistance 2–5 MΩ) mounted on the headstage of a DAGAN 8900 (Dagan Corporation, Minneapolis, MN, USA) patch-clamp amplifier, allowing cancellation of capacitative 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, USA) were used to acquire and analyze the data. Control and test solutions were changed using a multi-barreled concentration-clamp device (Gandía et al., 1995), the common outlet of which was placed within 100 μm of the cell to be clamped. The flow rate (0.2–0.5 ml min^{-1}) was regulated by gravity to achieve a complete replacement of the cell surroundings within less than 1 s. All experiments were performed at room temperature (22–24°C).

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

2.3. Catecholamine release from rat adrenal glands

Female Sprague-Dawley rats weighing 250–300 g from our local animal quarters were used. The animal experiments were approved by the Ethical Committees of La Laguna University and Universidad Autónoma de Madrid.

Catecholamine secretion experiments were conducted according to the technique described by Borges et al. (1986) adapted for the rat (Borges, 1993). Briefly, animals were anaesthetized with sodium pentobarbitone (50 mg/kg i.p.). Both adrenal glands were exposed and perfused in vitro through a cannula placed in the adrenal vein. Tissues were perfused with a Krebs-bicarbonate solution containing (in mM): NaCl 119, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25 and glucose 11; the pH was kept at 7.4 by bubbling with 95% O_2 and 5% $C0_2$. Glands were placed in plastic chambers and the perfusate passed through an electrochemical detector as described elsewhere (Borges et al., 1986). All the experiments were carried out at room temperature (22–24°C).

Pulses of secretagogue (histamine 30 μM, methacholine 30 μM or high K^+ 23.6 mM with isosmotic reduction of Na^+) were applied for 15 s every 8 min. When the secretory responses became stable, increasing concentrations of otilonium bromide were added to the perfusion buffer. Data were normalized as a percentage of initial control responses.

2.4. Materials

Otilonium bromide was a kind gift of Dr Carganico (Menarini, Barcelona, Spain). Collagenase A was from Boehringer Mannheim (Madrid, Spain). DMEM, fetal calf serum, penicillin and streptomycin were purchased from GIBCO (Madrid, Spain). Tetrodotoxin, methacholine, atropine, EGTA and histamine were purchased from Sigma Chemicals (UK) and Bay K 8644 (methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-

5-carboxylate) from RBI (Natick, MA, USA). Salts used in the preparation of buffers were reagent grade from Merck (Madrid, Spain).

2.5. Statistical analysis

Results are expressed as means ± S.E.M. The statistical differences between means of two experimental results were assessed by Student's *t*-test. A value of *P* equal or smaller than 0.05 was taken as the limit of significance.

3. Results

3.1. Effects of otilonium on whole-cell I_{Ba}

After a voltage-clamped cell (holding potential −80 mV) was entered, test potentials to 0 mV and 50 ms duration were continuously applied at 10–15 s intervals. As the cytosol dialysis progressed, I_{Ba} (10 mM external Ba^{2+}) increased from an initial value of about 200 pA to a stable plateau value of 441 ± 30 pA ($n = 39$ cells). After stabilization of I_{Ba} (usually 3–5 min), 10 μM otilonium was externally applied and removed, as shown by the horizontal bar in Fig. 2A. It is seen that I_{Ba} was depressed 67% upon drug application. The current remained depressed as long as otilonium was being superfused. Removal of the drug led to an almost complete recovery of I_{Ba}. Note that current recovery had an initial rapid phase followed by a slower phase. It seemed, therefore, as if otilonium partly remained attached to its inhibitory site on Ca^{2+} channels. Otilonium inhibited the peak I_{Ba} in a concentration-dependent manner (Fig. 2B). The whole-cell current was blocked more than 90% by 30 μM otilonium. The IC_{50} for inhibition was 4.7 μM.

At 1 μM, otilonium did not change the kinetics of activation, inactivation or deactivation of I_{Ba}. This is seen in Fig. 3, where full peak current-voltage (*I-V*) curves in the absence (control) and the presence of 1 μM otilonium are shown. No shifts of this curve were observed (see dashed *I-V* curve of current blocked by otilonium). Otilonium blocked I_{Ba} at all potentials tested, without significantly altering the time course of the current (see original traces in the insets to Fig. 3).

3.2. Effects of otilonium on I_{Ba} when given intra- or extracellularly

Since otilonium has a quaternary ammonium group, its intramembrane diffusion to its binding site on the Ca^{2+} channel might considerably differ if it is given extra- or intracellularly. Fig. 4 shows an experiment aimed at testing this hypothesis.

A rat chromaffin cell was sealed with a patch pipette; the intracellular solution contained 10 μM otilonium. After the seal was ruptured, I_{Ba} grew to reach a stable

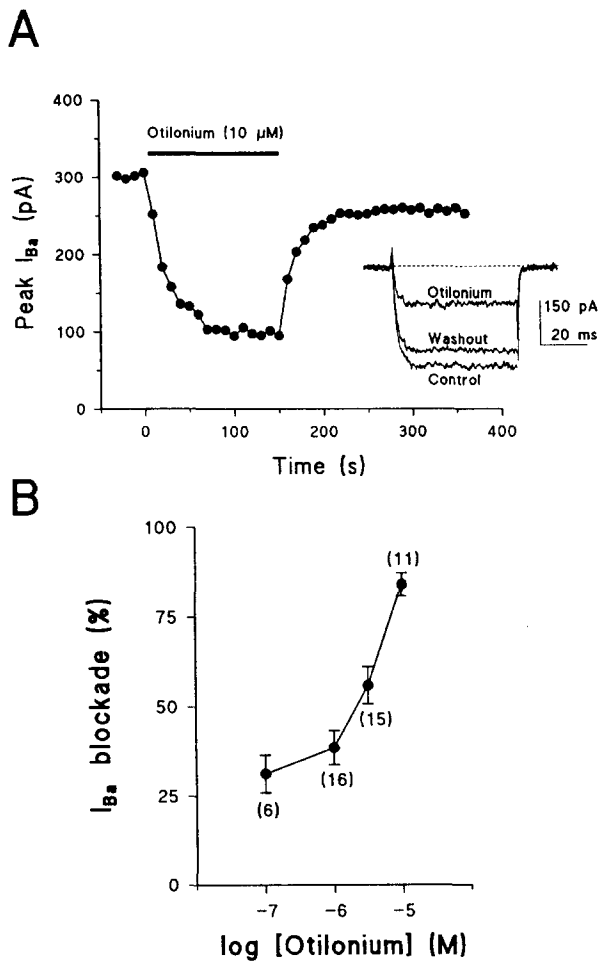

Fig. 2. (A). Time course of I_{Ba} induced by application of 50 ms test pulses to 0 mV every 15 s, to a voltage-clamped rat chromaffin cell whose membrane potential was held at −80 mV. Recording was started after a stabilization period of 2–3 min and otilonium was superfused at time 0 for the time indicated by the horizontal bar. (B) Concentration dependence of the blockade of I_{Ba} peak induced by otilonium. Data were obtained by pooling the results obtained in various cells. Cells were held at −80 mV and a 50 ms depolarizing pulse to 0 mV was applied either before or after the superfusion of the cells with a solution containing otilonium at different concentrations. Data were normalized with respect to control values recorded in the absence of otilonium; they are expressed as means ± S.E.M. of the number of cells indicated in parentheses.

plateau. After 5 min dialysis with otilonium, no changes in the plateau current were observed. Application of external otilonium (10 μM) produced a quick blockade of the current (Fig. 4A). After wash out of the drug, I_{Ba} partially recovered. Fig. 4B shows the time course of I_{Ba} in a control cell dialysed with an otilonium-free intracellular solution. The dialysis of the cell after seal rupture produced changes of I_{Ba} similar to those seen in cells dialysed with 10 μM otilonium. The external application of otilonium (10 μM) produced a similar blockade and recovery of the current. Thus, the maximum peak I_{Ba} in control cells was 364 ± 41 pA ($n = 14$ cells) and in cells dialysed with otilonium 435 ± 57 pA ($n = 20$ cells). Blockade of I_{Ba} by external otilonium was 67 ± 4% for control cells

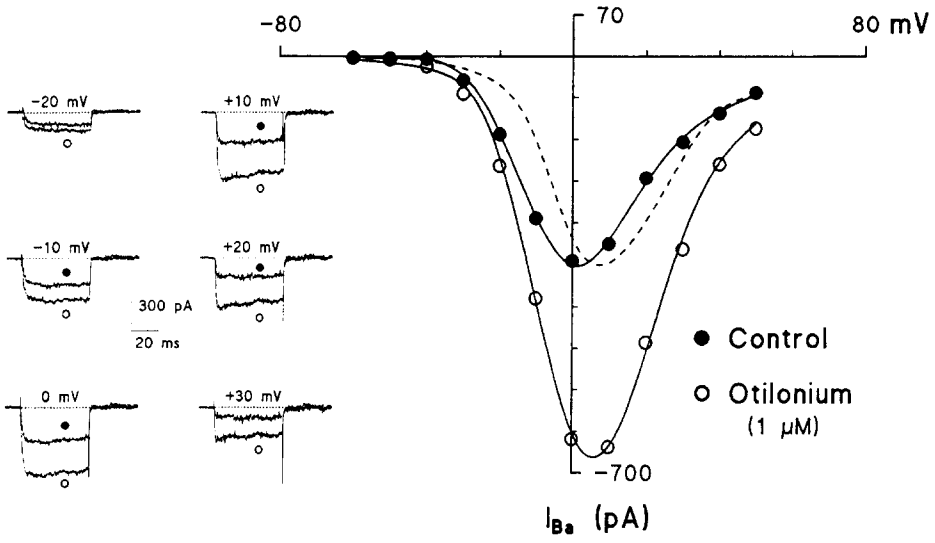

Fig. 3. Peak current-voltage (I/V) relationship for I_{Ba} recorded in a rat chromaffin cell before (control) and after superfusion of the cell with a solution containing otilonium (1 μM). Dashed line represents the current blocked by otilonium. Insets show capacitative and leak subtracted current traces obtained at the different test voltages indicated. Note that otilonium blockade was similar at all voltages tested, without significant changes in the activation and inactivation kinetics during the 50 ms depolarizing pulses.

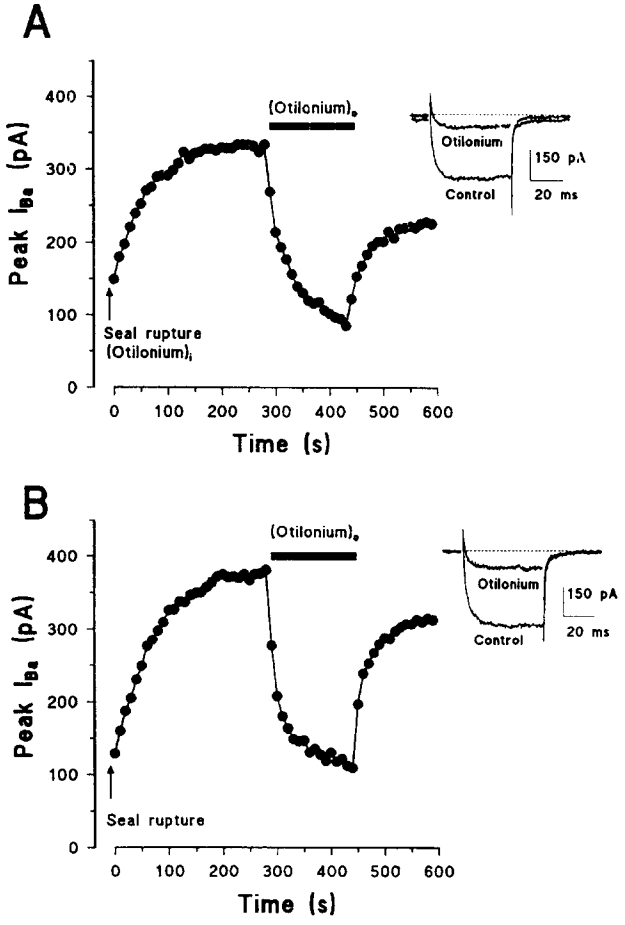

Fig. 4. Effects of otilonium on I_{Ba} when given intra- or extracellularly. In (A), the intracellular patch pipette solution contained 10 μM otilonium which entered the cell when the seal was broken (arrow). In (B), the cell was dialysed with the control intracellular solution. External otilonium (10 μM) was applied as shown by the horizontal bar. Insets show original traces immediately before addition and wash out of the extracellular otilonium, $(Otilonium)_o$.

and 72 ± 3% for cells dialysed with otilonium. After washing out the external otilonium, recovery of I_{Ba} reached 66 ± 5% of the initial current in control cells and 64 ± 4% in cells dialysed with otilonium.

3.3. Effects of otilonium on catecholamine release from intact perfused rat adrenal glands

To assess the antimuscarinic potency of otilonium, a concentration-response study was carried out on catecholamine release evoked by stimulation of adrenal glands with the pure muscarinic receptor agonist methacholine. These results were compared with those obtained with

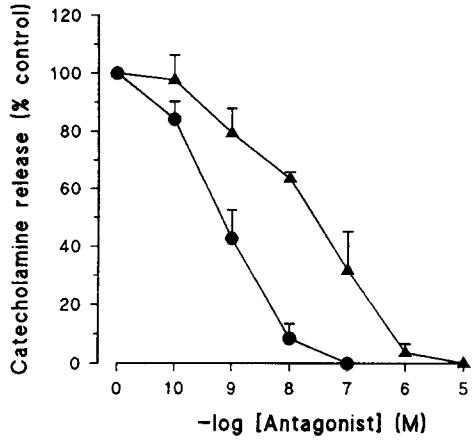

Fig. 5. Antimuscarinic effects of otilonium compared with atropine. Pulses of methacholine (30 μM) were applied for 15 s every 8 min. When secretory responses became stable, increasing concentrations of otilonium (triangles) or atropine (circles) were perfused to the gland. Data were normalized to the first four to five secretory peaks prior to muscarinic receptor antagonist application. Data show the means ± S.E.M. for four to five different glands.

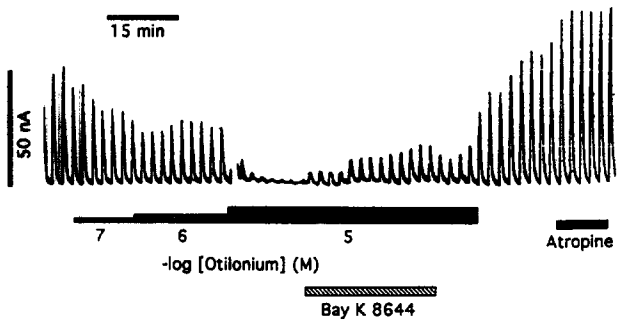

Fig. 6. Effects of otilonium bromide on K^+-evoked catecholamine release in the isolated rat adrenal. Secretion was continuously recorded as described in Methods. Pulses of high K^+ (23.6 mM) solution were applied for 10 s every 2 min. When secretory responses became stable, increasing cumulative concentrations of otilonium (10^{-7}–10^{-5} M) were perfused through the gland (shaded bar). Bay K 8644 was then added to the solution as indicated by the hatched bar (final concentration 10^{-7} M). After recovery of the response, atropine (10^{-5} M) was perfused (solid bar). The calibration bar on the left indicates the oxidation current in nA (approximately 450 ng/ml of catecholamines). Upper solid bar indicates the time scale. Traces show a typical experiment. Similar results were obtained in four rats.

muscarinic receptor antagonist atropine. Fig. 5 shows the inhibitory effects caused by cumulative concentrations of otilonium and atropine on the secretion evoked by short pulses of 30 μM methacholine. Atropine abolished the response at 10^{-7} M and otilonium at 10^{-5} M. The inhibition caused by these agents had an apparent IC_{50} of 0.5 and 20 nM, for atropine and otilonium, respectively. The methacholine secretion responses were restored upon 30 min superfusion with drug-free medium (not shown).

To test whether otilonium had actions other than the anticholinergic action, the effects of otilonium and atropine

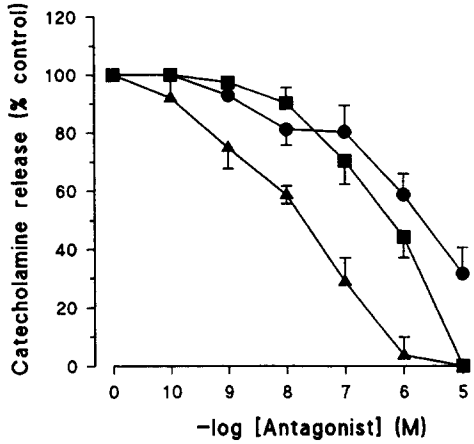

Fig. 7. Effects of otilonium on catecholamine secretion evoked by three different agents. Pulses of secretagogue were applied for 15 s every 8 min. When secretory responses became stable, increasing concentrations of otilonium were perfused through the glands. Secretagogues tested were methacholine, 30 μM (triangles); histamine, 30 μM (circles); and high K^+, 23.6 mM (squares). Data were normalized to the first four to five secretory peaks prior to otilonium application. Data show the means ± S.E.M. for five to eight different glands.

on secretion evoked by brief pulses of high K^+ (23.6 mM)-containing solutions were explored. Fig. 6 shows typical traces from an experiment with secretory responses evoked by 10 s pulses of high K^+ applied every 2 min. Cumulative concentrations of otilonium caused a progressive inhibition of secretion, which was abolished at 10^{-5} M. The L-type Ca^{2+} channel agonist Bay K 8644 (10^{-7} M) partially reversed the blocking effect of otilonium. At a concentration as high as 10 μM, atropine did not modify the catecholamine release evoked by K^+ (right part of Fig. 6).

Fig. 7 summarizes the effects of cumulative concentrations of otilonium on K^+-, methacholine- and histamine-evoked responses. The methacholine data are those of Fig. 5. The apparent IC_{50} for high K^+ and histamine were 0.7 and 3 μM, respectively. In spite of these close values, both curves exhibited different slopes. Also, it is worth noting that a concentration of otilonium (10 μM) which abolished the secretion evoked by K^+ or methacholine did not completely block the release evoked by histamine.

4. Discussion

We have shown in this study that otilonium blocks whole-cell currents through Ca^{2+} channels in isolated rat adrenal chromaffin cells, and that the release of catecholamine triggered by methacholine, histamine or K^+ in perfused rat adrenal glands.

As in neurones (Olivera et al., 1994), cat (Albillos et al., 1994) and bovine chromaffin cells (Albillos et al., 1993), multiple types of voltage-dependent Ca^{2+} channels have been recently discovered in rat adrenal medulla chromaffin cells (Gandía et al., 1995). Half of the whole-cell Ba^{2+} current is accounted for L-type Ca^{2+} channels, N-type channels account for 30% of the current and P-type channels provide another 15%. Because otilonium inhibited almost completely the whole-cell I_{Ba} in rat chromaffin cells at the higher concentration used (10 μM), it seems clear that otilonium does not distinguish between Ca^{2+} channel subtypes. Thus, otilonium shares with compounds such as R56865 (*N*-[1-(4-(4-fluorophenoxy)butyl)]-4-piperidinyl-*N*-methyl-2-benzothiazolamine) (Gárcez-Do-Carmo et al., 1993), dotarizine and flunarizine (Villarroya et al., 1995) their wide-spectrum profile as blockers of various neuronal Ca^{2+} channel subtypes.

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 between the lipophilic Ca^{2+} channel blockers that is reflected in the rate of blockade and deblockade of the current. Thus, R56865, dotarizine and flunarizine induced a slowly developing blockade of I_{Ba} in bovine chromaffin cells. In addition, such blockade was readily reversible in the case of R56865 (Gárcez-Do-Carmo et al., 1993), more

slowly reversible in the case of dotarizine and hardly reversible with flunarizine (Villarroya et al., 1995). In contrast, blockade of I_{Ba} by otilonium was fully established after 10–20 s, especially at the 10 μM concentration, and was quickly reversible after wash out of the drug. It is worth noticing that in many cases the reversibility was only partial and some current remained blocked several minutes after wash out of otilonium. Another interesting feature related to the polarity of otilonium rests in its lack of blockade when given intracellularly through the patch pipette, suggesting that the compound has access to the Ca^{2+} channel-blocking sites only from the outside mouth.

The effects of otilonium on catecholamine release were investigated using three secretagogues: methacholine, histamine and K^+. Methacholine, a pure muscarinic receptor agonist in chromaffin cells (Borges et al., 1987) was used to test the antimuscarinic action of otilonium in these cells. It was clear that as in the gastrointestinal system, where otilonium blocks muscarinic receptors, the compound also inhibited the muscarinic-evoked secretion of catecholamines with a much higher potency than histamine and K^+, suggesting a direct action on muscarinic receptors and a higher affinity of otilonium for such receptors.

Histamine promotes the release of adrenal catecholamines in the rat adrenal through the activation of a histamine H_1 receptor subtype (Borges, 1994). We have used histamine as a secretagogue agent because when applied as a short stimulus (i.e. less than 20–30 s), the intracellular Ca^{2+} rise is partially caused by external Ca^{2+} entering through voltage-dependent Ca^{2+} channels; at least 50% of the Ca^{2+} rise, however, seems to come from intracellular stores (Borges, 1993). This could explain why otilonium is a less effective antagonist of the histamine responses and also why a component of secretion remains unaffected after perfusion of high concentrations of otilonium.

K^+-evoked secretory responses are independent of muscarinic or histamine receptors and are fully dependent on extracellular Ca^{2+}. This can explain why atropine did not affect the K^+-evoked release, suggesting that atropine lacks any Ca^{2+} channel-blocking effect, even at concentrations as high as 10^{-4} M. All the effects of otilonium on K^+-evoked secretion should be caused by blockade of Ca^{2+} entry. It is also possible that otilonium can have another target like intracellular proteins of the secretory machinery, but it is unlikely that a quaternary amine could penetrate into the cell. Also, the rapid reversible blockade caused by otilonium on K^+-evoked responses does not support the existence of an intracellular site of action for this drug. This idea is consistent with the fact that otilonium has a quaternary ammonium group and is therefore charged at all pH values, making difficult its diffusion through the cell plasma membrane, as discussed above.

Different types of Ca^{2+} channel antagonists are being developed to use clinically as spasmolytic agents for gastrointestinal diseases (Feron et al., 1992; Bobo et al., 1994). In the light of the present results, it seems that otilonium could be one of these Ca^{2+} channel blockers, and might exert its effects locally on the intestinal wall because of its poor systemic absorption, due to its high polarity. So, otilonium can produce these clinically useful effects at least through three mechanisms: (1st) a well established antimuscarinic action directly on intestinal smooth muscle; (2nd) blockade of postsynaptic L-type Ca^{2+} channels in such muscle; and (3rd) inhibition of presynaptic Ca^{2+} channels and the release of spasmogenic transmitters at the intestinal wall, i.e. acetylcholine, opiates, ATP. Experiments are being planned to test this hypothesis.

In conclusion, otilonium can be classified as a novel, wide-spectrum Ca^{2+} channel antagonist. Therefore, it can serve as a useful molecular model to develop more selective and powerful Ca^{2+} channel blockers, both at presynaptic neuronal terminals and at postsynaptic effector cells.

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