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Ca^{2+}-ACTIVATED K^+ CHANNELS MODULATE MUSCARINIC SECRETION IN CAT CHROMAFFIN CELLS

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SUMMARY

1. This study was aimed at testing the hypothesis that Ca^{2+}-dependent K^+ channels regulate the release of catecholamines mediated by muscarinic stimulation of cat adrenal chromaffin cells. Two parameters were measured: the secretory response to brief pulses of methacholine (100 μM for 10 s) in intact cat adrenal glands perfused at a high rate with oxygenated Krebs solution; and the changes in cytosolic Ca^{2+} concentrations, $[Ca^{2+}]_i$, produced by puff applications of methacholine pulses (also 100 μM for 10 s) in isolated single cat adrenal chromaffin cells loaded with Fura-2.

2. A pulse of methacholine released 805 ± 164 ng of catecholamines (mean of thirty-two pulses). *d*-Tubocurarine (DTC) increased the secretory response in a concentration-dependent manner. The maximum increase (around 1000 ng catecholamines over control values) was reached at 100 μM-DTC and the EC_{50} was around 10 μM.

3. The secretory responses to methacholine alone, or to the combination of methacholine plus DTC, were strongly dependent on the extracellular Ca^{2+} concentration, $[Ca^{2+}]_o$. Thus Ca_o^{2+} removal from the perfusing solution for 5–10 min abolished catecholamine release.

4. At 0·1 μM, isradipine (an L-type Ca^{2+} channel blocker) inhibited by 71% the secretory response to DTC plus methacholine. At 1 μM, Bay K 8644 (an L-type Ca^{2+} channel activator) increased 2-fold the secretory response to DTC plus methacholine (2746 ng of catecholamines).

5. Apamin (1 μM) increased 3·5-fold the secretory response to methacholine pulses (from 500 to 1800 ng of catecholamines).

6. Methacholine pulses enhanced $[Ca^{2+}]_i$ from the resting level of 100 nM to a peak of 1000 nM which quickly declined to basal level. DTC (100 μM) enhanced by 20% the $[Ca^{2+}]_i$ peak and substantially prolonged its duration.

7. Apamin (1 μM) increased by 60% the $[Ca^{2+}]_i$ peak evoked by methacholine, and delayed the initiation of decline of the $[Ca^{2+}]_i$ peak.

8. These results are compatible with the idea that muscarinic stimulation depolarizes the cat adrenal chromaffin cell through an unidentified mechanism.

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Depolarization is probably counteracted by activation of Ca_i^{2+}-dependent K^+ channels. Therefore, inhibition of these channels enhances depolarization and firing of action potentials which activate voltage-dependent L-type Ca^{2+} channels to increase further the Ca_i^{2+} signal and the secretory response. Thus Ca_i^{2+}-dependent K^+ channels, probably of the small-conductance type (SK), seem to be involved in the modulation of muscarinic-evoked catecholamine release responses in cat adrenal chromaffin cells.

INTRODUCTION

Since the pioneering observations of Dale (1914) and Feldberg, Minz & Tsudimura (1934), the perfused cat adrenal gland has been an excellent model to study the release of catecholamines in response to muscarinic stimulation of its chromaffin cells (Douglas & Poisner, 1965; Lee & Trendelenburg, 1967; Lee, 1972; Kirpekar, Prat & Schiavone, 1982; Borges, Sala & García, 1986; Borges, Ballesta & García, 1987; Ballesta, Borges, García & Hidalgo, 1989; Abad, Garrido, López & García, 1992). However, the mechanism underlying such a response remains still unknown.

Though Ca^{2+} from internal stores might somehow contribute a small proportion, most of the Ca^{2+} required to trigger and maintain the muscarinic secretion comes from the extracellular milieu (Abad *et al.* 1992). If so, the question is how external Ca^{2+} (Ca_o^{2+}) gains access, through the plasma membrane, to the internal secretory machinery. Four years ago we suggested that Ca_o^{2+} could enter the cell through an ionophore coupled to the muscarinic receptor (Borges *et al.* 1987). However, it could also be that Ca^{2+}-dependent K^+ channels are regulating the permeability to Ca_o^{2+} (and the secretory process) in chromaffin cells stimulated with muscarinic agonists. In fact, high-conductance, Ca^{2+}-dependent K^+ channels (BK) and low-conductance, Ca^{2+}-dependent K^+ channels (SK) have been identified and characterized in chromaffin cells (Marty, 1981; Marty & Neher, 1985; Artalejo & Neher, 1991).

In this study we tested the hypothesis that Ca^{2+}-dependent K^+ channels could be involved in the regulation of catecholamine release triggered by muscarinic stimulation of cat adrenal medulla chromaffin cells. We explored the cytosolic Ca^{2+} (Ca_i^{2+}) signals in cultured cat chromaffin cells stimulated with methacholine, and correlated them with secretory signals obtained in intact perfused cat adrenal glands. The proper use of various K^+ channel blockers suggests that both the Ca_i^{2+} and secretory signals are probably modulated by a Ca^{2+}-dependent K^+ channel activated by the increase in $[Ca^{2+}]_i$ provoked by muscarinic stimulation.

METHODS

Perfusion of adrenal glands

Cats of both sexes weighing 2·5–4 kg were anaesthetized by I.P. administration of sodium pentobarbitone (50 mg kg^{-1}). Both adrenal glands were removed and retrogradely perfused through the adrenal vein with Krebs–Tris solution having the following composition (mM): NaCl, 134; KCl, 5·9; $MgCl_2$, 1·2; $CaCl_2$, 2·5; glucose, 11, and tris-(hydroxymethyl)-aminomethane, 10. The solution was bubbled with pure O_2, the final pH being 7·4, at 37 ± 2 °C, and perfused at a rate of 6 ml min^{-1} through the gland. K^+-rich solutions (35 mM) were made by adding KCl to Krebs–Tris solution with a concomitant reduction of NaCl to maintain isotonicity. Zero-Ca^{2+} solutions were made up by removing Ca^{2+} from the Krebs–Tris solution. Experiments with 1,4-dihydropyridine derivatives were performed under sodium light.

Collection of perfusate samples

After 1 h of initial perfusion with Krebs–Tris solution, collection of perfusate samples at 30 s intervals was initiated. The first sample was collected to determine the spontaneous catecholamine output; then, the secretagogue (usually methacholine, 100 μM for 10 s) was perfused followed by the collection of two more 30 s samples. After a 15 min washout period with Krebs–Tris solution, another stimulus was applied and the same samples collected. Each gland was sequentially stimulated 5–10 times following this protocol.

To test their effects on secretion, drugs were present 2–10 min before, during the 10 s stimulation and 60 s thereafter. Samples were collected in iced assay tubes containing enough perchloric acid to give a final concentration of 0·05 M.

Catecholamine assay

The total catecholamine content of perfusate samples (noradrenaline plus adrenaline) was determined according to Shellenberger & Gordon (1971) without further purification on alumina. Catecholamines present in each collection tube were expressed as ng 30 s^{-1}. The net catecholamine release evoked by the different secretagogues during the pulse and the following 60 s was determined after subtracting the basal (spontaneous) release and expressed as ng $pulse^{-1}$. Appropriate blanks were made with those molecules suspected of having native fluorescence.

Isolation and culture of cat adrenal medulla chromaffin cells

Both adrenal glands from cats were prepared as described above. Through the cannula placed in the adrenal vein, the adrenals were injected three times with Ca^{2+}- and Mg^{2+}-free Locke NaOH buffer (154 mM-NaCl, 5·6 mM-KCl, 3·5 mM-$NaHCO_3$, 5·6 mM-glucose, and 10 mM-HEPES buffer at pH 7·2) at room temperature. Medullae digestion was achieved by injecting with a syringe 1 ml of a solution containing 0·25% collagenase, 0·5% bovine serum albumin, and 0·01% soybean trypsin inhibitor in Ca^{2+}–Mg^{2+}-free Locke buffer until the glands swelled well. Then, incubation at 37 °C for 15 min followed. This procedure was repeated thrice. Following this, the glands were cut longitudinally and the liquified medullae gently scrapped out from the cortex. The collagenase was washed out from the cells with large volumes of Ca^{2+}–Mg^{2+}-free Locke buffer. After washing 3 times, cells were resuspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum containing 10 μM each of cytosine arabinoside and fluorodeoxyuridine, 50 U ml^{-1} penicillin and 50 μg ml^{-1} streptomycin. Cells were plated on circular glass coverslips and incubated at 37 °C in a water-saturated, 5% CO_2/95% air atmosphere. The medium was changed 24 h later and then every second day.

Measurement of intracellular calcium transients in Fura-2-loaded cat chromaffin cells

Chromaffin cells were loaded with Fura-2 (Grynkiewicz, Poenie & Tsien, 1985) by incubating them with Fura-2/AM (4 μM) for 30 min at room temperature in a standard medium (pH 7·4) containing (mM): NaCl, 145; KCl, 5·9; $MgCl_2$, 1·2; $CaCl_2$, 2·5; sodium HEPES, 10; glucose, 10. The loading incubation was terminated by washing several times the coverslip with the cells attached in standard medium. Then, cells were incubated at 37 °C in the incubator for 15–30 min. The fluorescence of Fura-2 in single cells was measured with the photomultiplier-based system described by Neher (1989), which produces a spatially averaged measure of $[Ca^{2+}]_i$. Fura-2 was excited with a light alternating between 360 and 390 nm, using a Nikon 40 × objective. Emitted light was transmitted through a 425 nm dichroic mirror and 500–545 nm barrier filter before being detected by the photomultiplier. $[Ca^{2+}]_i$ was calculated from the ratios of the light emitted when the dye was excited by the two alternating excitation wavelengths (Grynkiewicz *et al.* 1985).

Statistical analysis

The statistical significance of differences between means was determined by Student's t test for paired or group data. Differences were considered significant at the level of $P < 0·05$.

Drugs and chemicals

Methacholine, *d*-tubocurarine (DTC), tetraethylammonium, hexamethonium, mecamylamine, pilocarpine, and the salts for the nutrient solutions were obtained from Sigma Chemical Co., Spain. Fura-2/AM was obtained from Molecular Probes, Eugene, OR, USA. Bay K 8644 was purchased from RBI, USA. Isradipine was obtained from Dr R. F. Hoff, Sandoz Laboratories, Basel, Switzerland.

RESULTS

The secretory response to methacholine

In Fig. 1*A*, the profile of catecholamine output in perfused cat adrenal glands stimulated with methacholine (100 μM for 10 s) is shown. The first point (zero time on the abscissa) represents the spontaneous release of catecholamines in the 30 s sample collected immediately before the pulse which amounted to 78 ± 7 ng 30 s^{-1}. Methacholine evoked a sharp increase of catecholamine release which peaked in the first sample collected, and then quickly declined in the two subsequent samples. Thus, the catecholamines released by the methacholine pulse were mostly recovered in the first 30 s sample collected.

Net catecholamine secretory responses (subtraction of basal release from the release obtained in the three 30 s samples collected) in thirty-two pulses of methacholine (100 μM for 10 s) amounted to 805 ± 164 ng $pulse^{-1}$ (mean $\pm$ S.E.M.). The secretory response to methacholine was fairly reproducible when applied several times to the same gland at 15 min intervals (Fig. 1*B*). This permitted the study of the effects of various concentrations of drugs in the same gland. It is worth noting that this secretory response to methacholine is purely mediated by muscarinic receptors, since it is fully blocked by submicromolar concentrations of atropine (Ballesta *et al.* 1989; Abad *et al.* 1992). The concentration of methacholine (100 μM) and the duration of the pulse (10 s) were selected on the basis of previous experiments using various stimulation parameters which suggested that these were adequate conditions to study the modulation by drugs of the secretory signal. With the manual procedure used here for the application of methacholine pulses, errors from stimulus to stimulus smaller than 10% were detected.

d-Tubocurarine potentiates the catecholamine secretory response to methacholine

DTC inhibits a Ca^{2+}-dependent K^+ conductance in bullfrog sympathetic ganglion cells (Nohmi & Kuba, 1984), an outward K^+ current induced by Ca^{2+} injections in mouse neuroblastoma $\times$ rat glioma hybrid NG108–15 cells (Brown & Higashida, 1988) and Ca^{2+}-mediated K^+ efflux from guinea-pig hepatocytes (Cook & Haylett, 1985). Since these effects were observed at low micromolar concentrations, DTC seemed to be a good tool to test the hypothesis raised in this study with the following experiment.

A given gland was stimulated six times with methacholine (100 μM for 10 s) at 15 min intervals. The first stimulus explored the initial size of the secretory signal. The subsequent four stimuli were given in the presence of increasing concentrations of DTC. Each concentration was present since 5 min before, during the pulse and along the three 30 s collection samples. Figure 2 shows that DTC increased the secretory signal in a concentration-dependent manner. At 10^{-6} M, a visible enhancement of the response was already present. At 10^{-5} M, secretion was doubled (1646 ± 171 ng) and at 10^{-4} M the secretory signal was further increased to 1966 ± 232 ng. A rough graph calculation gave a figure of around 10 μM for the EC_{50} of DTC to enhance the muscarinic response. This is in the range of the values of the concentrations of DTC required to block Ca^{2+}-dependent K^+ channels (Nohmi & Kuba, 1984; Cook & Haylett, 1985).

Effects of nicotinic receptor blockers other than d-tubocurarine on methacholine-evoked secretion

DTC (Abad *et al.* 1992), like hexamethonium (Jaanus, Miele & Rubin, 1967) and mecamylamine (Borges *et al.* 1986), are known to block the nicotinic-mediated

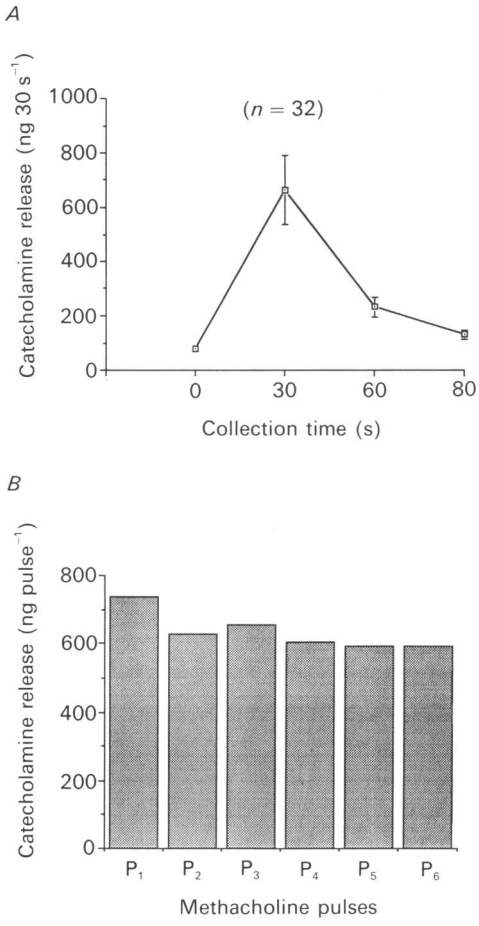

Fig. 1. *A*, profile of catecholamine release in response to a 10 s pulse of methacholine (100 μM) given to cat adrenal glands perfused with oxygenated Krebs–Tris solution. After collecting a prepulse 30 s sample (0 s on abscissa) to determine the basal rate of secretion, three 30 s samples were collected: the first was collected during the 10 s stimulation period with methacholine plus 20 s in Krebs–Tris, and the second and third samples were collected also in Krebs–Tris solution for 30 s. Data are means $\pm$ S.E.M. of thirty-two methacholine pulses from different glands. *B*, net catecholamine release evoked by subsequent pulses of methacholine (100 μM during 10 s) given to individual glands at 15 min intervals. Net catecholamines represent the area under the curve in *A*, after subtracting the basal release immediately preceding each pulse, from each of the three 30 s samples collected after every methacholine pulse. Data are from a typical experiment.

secretory response in the cat adrenal medulla. Therefore, the possibility arose that the potentiation of methacholine responses could be associated with nicotinic receptor interaction. This did not seem to be the case since concentrations of hexamethonium and mecamylamine ranging between 10^{-7} M and 10^{-4} M (which block

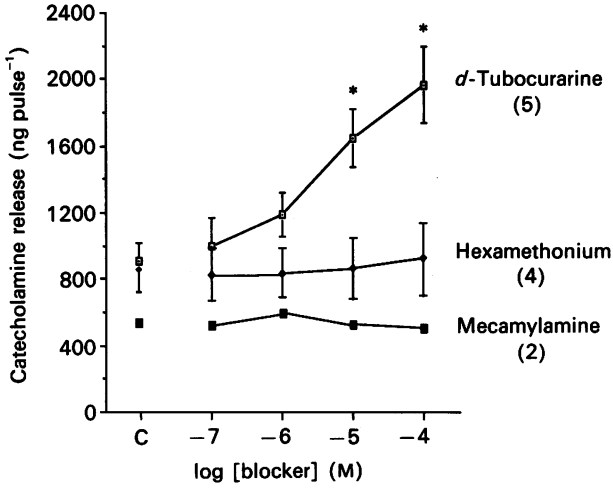

Fig. 2. Effects of *d*-tubocurarine (DTC), hexamethonium and mecamylamine on the release of catecholamines evoked by methacholine. Every compound was tested in separate glands. Each gland was stimulated five times with methacholine (100 μM for 10 s). Each drug concentration was present since 5 min before, during methacholine stimulation and during the ensuing sample collection, to recover the released catecholamines. Data are expressed as nanograms net total catecholamines released (evoked minus basal) in each methacholine test pulse. In the case of DTC and hexamethonium, data are means ± S.E.M of the number of glands shown in parentheses. The mecamylamine data are means of two glands. $*P < 0{\cdot}01$ with respect to control secretion (C).

fully the nicotinic receptor-mediated response) did not affect the release of catecholamines produced by methacholine pulses (Fig. 2). This result agrees with the observation that hexamethonium was much less potent than DTC in blocking Ca^{2+}-mediated K^+ efflux from guinea-pig hepatocytes (Cook & Haylett, 1985).

The experiment of Fig. 3 was designed to explore the possible interaction between DTC, hexamethonium and mecamylamine, as well as the reversibility of the effects of DTC. A gland was stimulated 8 times with methacholine pulses (100 μM for 10 s) at 15 min intervals. P_1 represents the control secretory signal to methacholine. P_2 and P_3 show that neither hexamethonium nor mecamylamine (100 μM each) affected the secretion response; however, DTC (100 μM) enhanced the secretion 4-fold (P_4). P_5 demonstrates that the DTC effect was readily reversible after 15 min washout with fresh Krebs–Tris solution. P_6 and P_7 show that the combined presence of DTC with hexamethonium, or with hexamethonium plus mecamylamine, did not potentiate further the methacholine response. Finally, P_8 illustrates once more the full reversibility of the DTC effects upon washout of the drug.

Calcium dependence of the secretory response evoked by the combination of d-tubocurarine and methacholine

Muscarinic-mediated secretory responses in the perfused cat adrenal gland are strongly dependent on extracellular Ca^{2+} (Ca_o^{2+}; Borges *et al.* 1987; Ballesta *et al.* 1989; Abad *et al.* 1992). It was therefore important to know whether the potentiation of the methacholine signal by DTC also involved Ca_o^{2+}. The following experiment suggests that this is so.

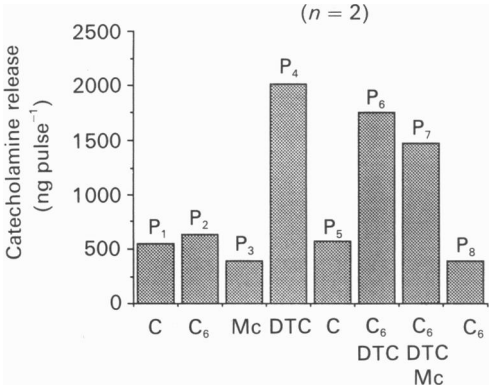

Fig. 3. Effects of the combined use of hexamethonium (C_6), mecamylamine (Mc) and *d*-tubocurarine (DTC) on the release of catecholamines evoked by pulses of methacholine (100 μM for 10 s). Glands were sequentially stimulated with methacholine pulses (100 μM for 10 s) at 15 min intervals 8 times (P_1–P_8). P_1, P_5 and P_8 are secretory responses to methacholine in the absence of other drugs (C, control secretion). The rest of the columns represent the secretion in the presence of 100 μM of the drug, or drug combination shown at the bottom of each column. Drugs were present 5 min before, during the methacholine pulse and during the collection of the subsequent 30 s samples. Data represent the mean net secretory responses obtained in two separate glands.

A gland was stimulated 4 times, at 15 min intervals, with methacholine pulses (100 μM for 10 s, P_1–P_4). Around 400 ng of catecholamines were secreted in the initial control pulse (P_1 in Fig. 4). In P_2, DTC (100 μM) potentiated this signal by about 2-fold. Removal of Ca^{2+} from the perfusion solution produced the practical abolition of the secretory responses to methacholine alone (P_3) and to methacholine in the presence of DTC (P_4). On returning to normal $[Ca^{2+}]_o$, the secretory responses to methacholine or the combination of DTC plus methacholine were re-established (not shown).

Effects of isradipine and Bay K 8644 on the potentiation by d-tubocurarine of muscarinic-mediated catecholamine release

If Ca_o^{2+} ions are critical in controlling the secretory response to methacholine plus DTC, one possibility is that upon muscarinic stimulation in the presence of DTC Ca_o^{2+} gains access to the secretory machinery through a dihydropyridine (DHP)-sensitive Ca^{2+} pathway. Experiments using isradipine (a selective L-type Ca^{2+} channel blocker; Hof, Scholtysik, Loutzenhiser, Vuorela & Neumann, 1984) and

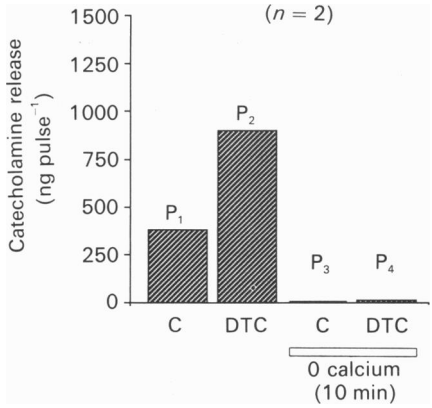

Fig. 4. The influence of external Ca^{2+} ions on the potentiation by *d*-tubocurarine (DTC) of the methacholine secretory response. Four pulses of methacholine (100 μM for 10 s) were sequentially applied to a given gland at 15 min intervals (P_1–P_4 on top of the columns). Pulse P_1 was given in the absence of any manipulation (control secretion). Pulse P_2 shows the enhanced secretory signals in the presence of 100 μM-DTC. These tests were repeated upon removal of Ca^{2+} from the perfusing solution for 10 min (P_3 and P_4). Data represent the net release of total catecholamines evoked by each methacholine pulse.

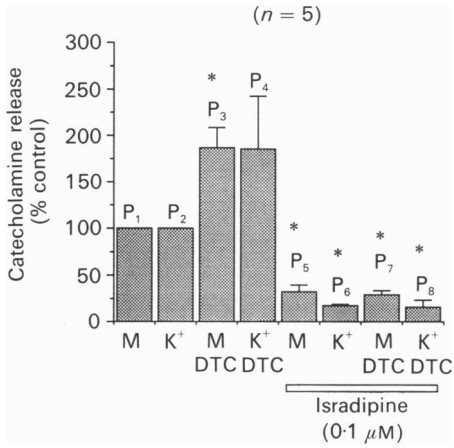

Fig. 5. Effects of isradipine on catecholamine release responses to methacholine (100 μM for 10 s) or K^+ (35 mM for 10 s). Four methacholine pulses were alternated with four K^+ pulses. Secretion obtained in the first two pulses was normalized to 100%; secretion in the six last pulses was expressed as a percentage of their respective (methacholine or K^+) initial secretory signal. The number of each pulse (given at 20 min intervals) is shown on top of each column; the experimental conditions are shown at the bottom of each column (M, methacholine; K^+, potassium; DTC, 100 μM-*d*-tubocurarine). Note that isradipine was present continuously during the four last pulses. Data are means ± S.E.M. of the number of glands shown in parentheses. $*P < 0.01$ compared with their respective methacholine or K^+ controls.

Bay K 8644 (an L-type Ca^{2+} channel activator; Schramm, Thomas, Towart & Franckowiak, 1983) suggest that this is so.

In the experiment of Fig. 5, each gland received alternating pairs of pulses with methacholine (100 μM for 10 s) and K^+ (35 mM for 10 s). The initial secretory response

to methacholine amounted to 924 ± 109 ng (P_1); K^+ secreted 2466 ± 916 ng of catecholamines. The initial secretory signals to methacholine and K^+ were normalized to 100%, and the rest of the pulses were expressed as percentages of these initial responses. DTC (100 μM) enhanced to 187 ± 22% and to 186 ± 57% the signals to

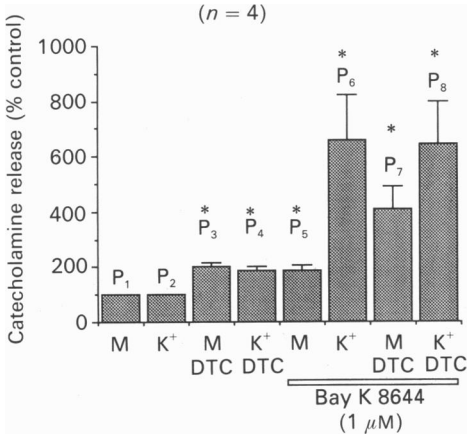

Fig. 6. Effects of Bay K 8644 on the release of catecholamines evoked by pulses of methacholine (M, 100 μM for 10 s) or high K^+ (35 mM for 10 s). The protocol here was similar to that of Fig. 5, except that Bay K 8644 (1 μM) was used instead of isradipine, during the last four secretory pulses (horizontal bar at the bottom of the figure). The number of each pulse (given at 20 min intervals) is shown on top of each column; the experimental conditions are reflected in the bottom of each column. (M, methacholine; K^+, potassium; DTC, 100 μM-*d*-tubocurarine). Data are means $\pm$ S.E.M. of the number of glands shown in parentheses. $*P < 0{\cdot}01$ compared with their respective methacholine or K^+ controls.

methacholine and K^+, respectively (P_3 and P_4 in Fig. 5). The next two pairs of pulses were applied in the continued presence of isradipine (10^{-7} M). In these conditions, the secretory responses were decreased to 32 ± 8% (methacholine) and to 18 ± 2% (K^+). When the secretagogues were applied in the presence of DTC, the signals were decreased to 29 ± 4% (methacholine) and to 16 ± 7% (K^+).

In Fig. 6, a similar experiment using Bay K 8644 instead of isradipine is shown. Bay K 8644 (10^{-6} M) was continuously present during the last four pulses. This DHP agonist enhanced the methacholine response to 188 ± 20% (P_5) and that to K^+ to 661 ± 162% (P_6). It is interesting to note that the potentiation by Bay K 8644 of the response to methacholine plus DTC was enhanced substantially more (407 ± 82%) than when methacholine was used alone (compare P_7 with P_5 in Fig. 6). Finally, the enhancement by Bay K 8644 of the secretory response to the combined use of DTC plus K^+ was similar (646 ± 154%) to that obtained when K^+ was used alone.

In conclusion, it seems that the K^+ secretory responses are substantially more sensitive to DHP than those of methacholine alone. However, the behaviour of Bay K 8644 with respect to the combined response to DTC plus methacholine closely resembles the K^+ response. It looks as if DTC were sensitizing the muscarinic

response to the potentiating effects of Bay K 8644, probably because DHP-sensitive Ca^{2+} channels are being recruited better under these conditions than when methacholine is used alone.

Effects of apamin on methacholine-induced secretion

As DTC, apamin is a potent inhibitor of K^+ currents induced by Ca^{2+} injections in NG108-15 cells (Brown & Higashida, 1988) and of a Ca^{2+}-dependent K^+ conductance

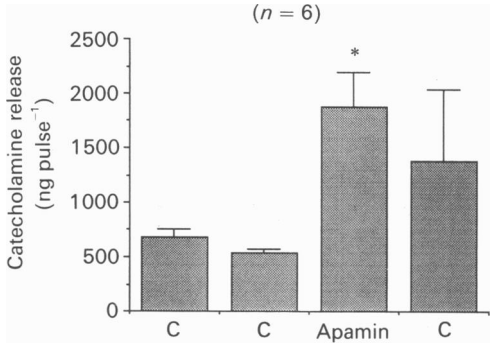

Fig. 7. Effects of apamin on catecholamine release responses to methacholine pulses (100 μM for 10 s). Glands were challenged with three methacholine pulses 15 min apart. The first pulse explored the control secretory response (C), the second was given in the presence of apamin (10^{-6} M, present since 2 min before, during the methacholine pulse and during the collection of the three 30 s samples), and the third pulse was applied 15 min after washing out apamin. $*P < 0{\cdot}01$ compared with their respective initial controls.

in rat sympathetic neurones (Kawai & Watanabe, 1986). In addition, we have recently found apamin to be a selective inhibitor of SK currents in chromaffin cells (Artalejo & Neher, 1991). Therefore, it was interesting to study whether apamin affected the muscarinic-mediated secretory response with the following experiment.

After equilibration, glands were challenged with two pulses of methacholine (100 μM for 10 s) which produced secretory responses of around 500 ng (Fig. 7). In the presence of apamin (10^{-6} M, present since 2 min before the pulse), the secretory response increased 5-fold (to around 2000 ng). On returning to an apamin-free medium, the methacholine response was still elevated (1379 ± 658 ng). Thus, apamin caused a drastic and slowly reversible enhancement of the catecholamine response triggered by muscarinic receptor activation.

Effects of tetraethylammonium on the secretory signal produced by methacholine

BK channels are blocked by the tetraethylammonium ion (TEA), a classical blocker of K^+ channels in various cell types (Blatz & Magleby, 1987). This is also true for chromaffin cells (Marty & Neher, 1985). Therefore, we decided to explore the effects of TEA on the secretory responses to methacholine pulses (100 μM for 10 s given at 15 min intervals to each individual gland).

Concentrations of 1–10 mM of TEA did not modify the secretory responses to

methacholine (Fig. 8). However, the concentrations 10–20 mM potentiated markedly the secretory signal. Thus, at 30 mM-TEA, the release of catecholamines evoked by methacholine was increased 4-fold over the control, initial values of secretion. This effect was readily reversible upon washing out the gland with a TEA-free Krebs–Tris solution.

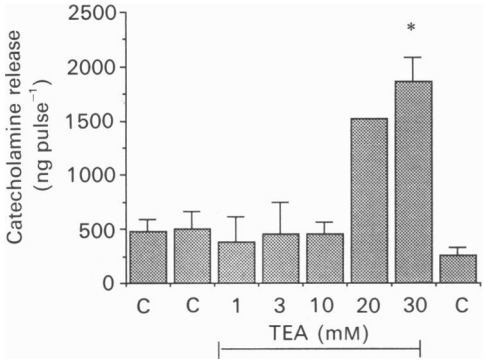

Fig. 8. Effects of tetraethylammonium (TEA) on the release of catecholamines evoked by methacholine pulses (100 μM for 10 s) given at 15 min intervals. The initial rate of methacholine-evoked secretion is indicated by the first two columns (C, control secretion). Each concentration of TEA was present 5 min before, during the methacholine pulse and during the post-stimulation sample collection. At the end of the experiment, a new control test stimulus was applied in order to see the reversibility of the TEA effects. Data are means $\pm$ S.E.M. of three glands; the datum for the 20 mM-TEA concentration is from a single gland. $*P < 0{\cdot}01$ with respect to the initial controls.

Effects of potassium channel blockers on the rate of spontaneous catecholamine release

Ca^{2+}-dependent K^+ channels may contribute to the repolarization phase of the action potential and to the regulation of prolonged repetitive discharges of action potentials. Since spontaneous action potentials are seen in chromaffin cells (Brandt, Hagiwara, Kidokoro & Miyazaki, 1976) it is likely that K^+ channel blockers could enhance the rate of action potential firing and the rate of catecholamine release.

In Fig. 9, experiments aimed at testing this possibility were performed. After equilibration of the glands two 30 s samples were collected to estimate the rates of spontaneous catecholamine outputs, which were around $40 \pm 3{\cdot}8$ ng 30 s^{-1} ($n = 26$). Then DTC (100 μM), apamin (1 μM) or TEA (30 mM) were introduced into the perfusion system for 5 min, and 30 s samples continuously collected. Neither DTC nor apamin increased the spontaneous rate of catecholamine release. In contrast, TEA increased 20-fold the initial rate of catecholamine output. After an initial secretory peak (around 2000 ng 30 s^{-1}) the rate of catecholamine release declined to a plateau at around 700 ng 30 s^{-1}. Stimulation by TEA of catecholamine release from perfused cat adrenals was previously reported by Kirpekar, Prat & Schiavone (1983); they attributed this effect to depolarization by TEA of chromaffin cells which, as demonstrated later on, caused marked fluctuations in cytosolic Ca^{2+} concentrations (Sorimachi, Yamagami & Nishimura, 1990).

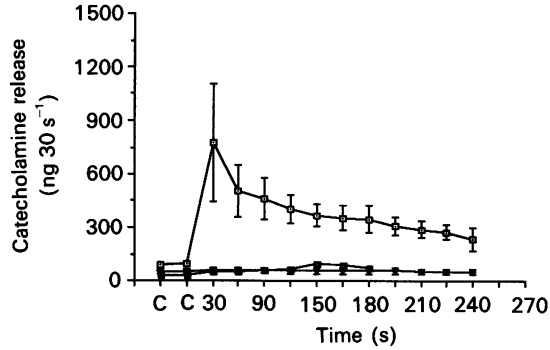

Fig. 9. Effects of *d*-tubocurarine (◆, $n = 7$), apamin (■, $n = 2$) and tetraethylammonium (⊡, $n = 6$) on the spontaneous release of catecholamines. After equilibration, glands were given *d*-tubocurarine (100 μM) or TEA (30 mM) for 4 min. Samples were continuously collected at 30 s intervals since 30 s before (basal catecholamine release) and during the period of exposure to each drug. Data are means ± S.E.M. of three glands.

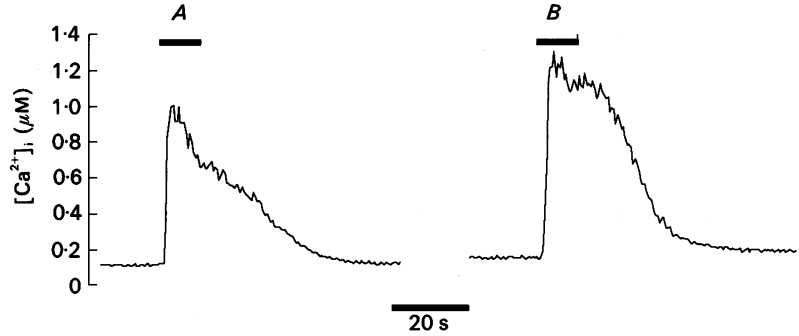

Fig. 10. Cytosolic Ca^{2+} transients in a single cat adrenal chromaffin cell sequentially stimulated with two pulses of methacholine (100 μM for 10 s) separated by 15 min. The cell was stimulated using a micropipette loaded with methacholine and placed near the cell surface. The first pulse was given in the absence of *d*-tubocurarine (*A*) and the second (*B*) in its presence (DTC, 100 μM, present 5 min before and during the methacholine pulse). Top horizontal bars reflect application of methacholine; the bottom horizontal bar is time.

Effects of d-tubocurarine and apamin on cytosolic Ca^{2+} signals evoked by methacholine

The hypothesis raised in this study implies that Ca^{2+}-dependent K^+ channels might shape the methacholine secretory profile. Therefore, the activation of such channels should be a consequence of an increase in $[Ca^{2+}]_i$ following methacholine application. Indeed, methacholine (puff application of a 10 s pulse of a 100 μM concentration) caused a sharp increase of $[Ca^{2+}]_i$ in single cat adrenal chromaffin cells (Fig. 10). This peak reached 1 μM and then declined gradually to the basal $[Ca^{2+}]_i$ of around 100 nM in the 30 s period following the methacholine pulse.

DTC (100 μM), superfused over the coverslip with the attached cell for 5 min, did not modify the basal levels of Ca_i^{2+}. However, DTC clearly modified the profile of the

$[Ca^{2+}]_i$ signal produced by methacholine (Fig. 10*B*). The $[Ca^{2+}]_i$ peak obtained during the first pulse was slightly higher (around 1300 nM) than the control peak. But the principal difference resides in the declining phase of the $[Ca^{2+}]_i$ change; after the peak, $[Ca^{2+}]_i$ remained elevated even for 10 s after the methacholine pulses ended.

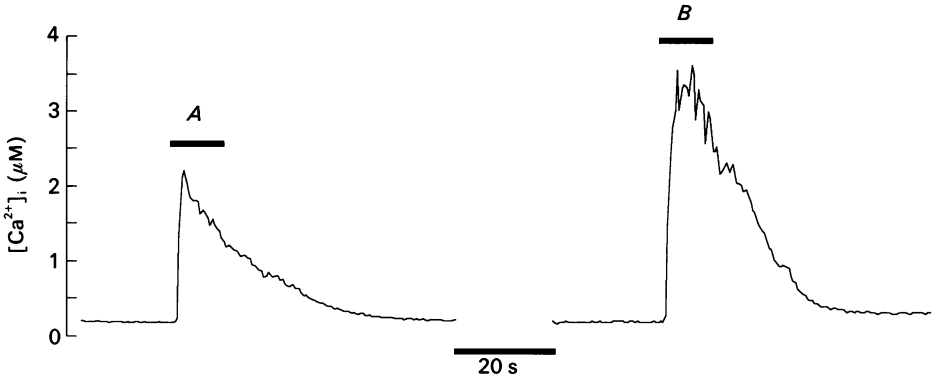

Fig. 11. Cytosolic Ca^{2+} transients in a single cat adrenal chromaffin cell sequentially stimulated with two pulses of methacholine (100 μM for 10 s) separated by 15 min. Experimental protocol as in Fig. 10 except for the use of apamin instead of *d*-tubocurarine.

Then, $[Ca^{2+}]_i$ started to decline even more quickly than under control conditions. This effect of DTC was reversible. Ten minutes after superfusion of the cells with DTC-free solution, the methacholine pulses given 1 min apart produced $[Ca^{2+}]_i$ traces similar to the initial control signals (not shown).

The effects of apamin on methacholine-evoked $[Ca^{2+}]_i$ transients are shown in Fig. 11. The first methacholine pulse produced an increase in $[Ca^{2+}]_i$ which peaked at around 2 μM, followed by the usual declining phase without delay (Fig. 11*A*). Apamin (10^{-6} M) did not change the basal $[Ca^{2+}]_i$. However, it produced a pronounced increase of the $[Ca^{2+}]_i$ peak to 3·5 μM in response to the application of methacholine (Fig. 11*B*). Note that the plateau phase after the peak appeared again, as in the case of DTC; after the plateau, the $[Ca^{2+}]_i$ signal gradually declined to basal levels. Note also that the oscillations of $[Ca^{2+}]_i$ were larger in the presence of apamin than in its absence. After 15 min of washing out apamin, the $[Ca^{2+}]_i$ peaks remained elevated to similar levels (not shown). These prolonged effects of apamin agree with the slow reversibility of its potentiating effects on secretion in the perfused cat adrenal gland.

DISCUSSION

Two main observations emerge from this study. First, DTC and apamin enhance the release of catecholamines evoked by methacholine stimulation of perfused cat adrenal glands. And second, they increase also the methacholine-induced $[Ca^{2+}]_i$ transients in single cat adrenal chromaffin cells loaded with Fura-2. Because these drugs selectively block Ca^{2+}-dependent K^+ channels in various cell types (Nohmi & Kuba, 1984; Cook & Haylett, 1985; Smart, 1987; Brown & Higashida, 1988) and

Ca^{2+}-dependent K^+ channels of various classes have been described in chromaffin cells (Marty, 1981; Marty & Neher, 1985; Artalejo & Neher, 1991), a link between muscarinic receptors and such channels might exist in the cat chromaffin cells. Those channels could modulate the muscarinic secretory response in the following frame.

(i) Muscarinic receptor stimulation causes cell depolarization, firing of action potentials, recruitment of voltage-dependent Ca^{2+} channels, a rapid increase of $[Ca^{2+}]_i$ and the triggering of catecholamine release.

(ii) Activation by raised $[Ca^{2+}]_i$ of Ca^{2+}-dependent K^+ channels counteracts the muscarinic depolarization, self-limiting in this manner the further firing of action potentials. Thus, this 'repressor' mechanism is limiting the muscarinic-mediated secretory response, which will be potentiated through the blockade of such channels by apamin or DTC.

Action of muscarinic receptors on the electrical properties of the chromaffin cell plasmalemma

The effects of muscarinic receptor agonists on the electrical properties of chromaffin cells are scarcely studied. Both inward and outward currents have been reported to be associated with stimulation of those cells with muscarinic agonists. Kubo & Kidokoro (1989) observed a slow outward K^+ current in response to muscarine in rat adrenal chromaffin cells voltage clamped at 0 or -20 mV. In contrast, three other studies arrived at the conclusion that muscarinic stimulation activated inward depolarizing currents. Knight & Maconochie (1987) suggested that muscarinic receptors depolarize the chicken chromaffin cells by opening non-selective cation channels. Akaike, Mine, Sasa & Takaori (1990) concluded that muscarine induced a slow depolarization and an apparent inward current in rat adrenal chromaffin cells, through the suppression of K^+ channels that are open at potentials near the resting membrane potential. Finally, Inoue & Kuriyama (1991) found that muscarine and oxotremorine produce an inward current associated with an increase in current noise at a holding potential of -40 mV in guinea-pig adrenal chromaffin cells; they conclude that the muscarinic receptor is coupled, through a GTP-binding protein, to a cation channel which is permeable to Na^+, K^+ and Ca^{2+}.

It is our opinion that muscarinic stimulation generates a depolarizing signal which triggers the firing of action potentials similar to those caused by muscarine in rat chromaffin cells (Akaike *et al.* 1990). This observation is in line with a previous report (Ladona, Aunis, Gandía & García, 1987) showing that Bay K 8644 almost tripled the peak secretory response to muscarine in perfused cat adrenal glands. It also agrees with the present study where a doubling of methacholine-evoked secretion of Bay K 8644 and a blockade by isradipine were observed. Since Bay K 8644 potentiates the release of catecholamines by increasing Ca_o^{2+} entry through L-type Ca^{2+} channels in chromaffin cells (García, Sala, Reig, Viniegra, Frias, Fonteríz & Gandía, 1984) and isradipine blocks it (Gandía, López, Fonteríz, Artalejo & García, 1987), it seems logical to implicate those channels in the regulation of the overall secretory response secondary to muscarinic stimulation. The channels will be recruited by the firing of action potentials evoked by methacholine in our present experimental conditions.

Muscarinic-mediated cytosolic calcium transients

To our knowledge, this is the first study where $[Ca^{2+}]_i$ transients secondary to muscarinic receptor activation have been measured in chromaffin cells from an animal species (i.e. the cat) which responds with healthy secretory responses to muscarinic agonists. Because of the easy production of large quantities of chromaffin cells, the bovine adrenal gland has been amply used to perform a wide variety of studies. In spite of the fact that these cells exhibit a poor (O'Sullivan & Burgoyne, 1989; Kim & Westhead, 1989) or a non-existent secretory response to muscarinic stimulation (Fisher, Holz & Agranoff, 1981; Almazan, Aunis, García, Montiel, Nicolás & Sánchez-Garcia, 1984; Livett & Boksa, 1984; Cheek & Burgoyne, 1985; Ballesta *et al.* 1989), an increase in $[Ca^{2+}]_i$ has been shown upon their stimulation with methacholine (Cheek, O'Sullivan, Moreton, Berridge & Burgoyne, 1989) or muscarine (Kim & Westhead, 1989; O'Sullivan, Cheek, Moreton, Berridge & Burgoyne, 1989). However, the methacholine (300 μM)-induced increase in $[Ca^{2+}]_i$ observed in single bovine adrenal chromaffin cells loaded with Fura-2 was only around 200 nM (Cheek *et al.* 1989). In cat chromaffin cells, a 3-fold lower concentration of methacholine caused increases of $[Ca^{2+}]_i$ ranging between 1000 and 2000 nM. On the basis of their observations Cheek *et al.* (1989) concluded that 'in response to the ineffective secretagogues methacholine and muscarine, the rise in Ca_i^{2+} originated only in one pole of the cell and even at the peak of the response Ca^{2+} was still generally restricted to this same area of the cell'. It seems clear that this reasoning does not apply to the feline chromaffin cell which responds to muscarinic stimulation with $[Ca^{2+}]_i$ transients and secretory responses much healthier than those of the bovine chromaffin cell. We believe that the modifications of the electrical properties of the plasmalemma exerted by muscarinic agonists, rather than specific spatial distribution of $[Ca^{2+}]_i$ transients, are responsible for the drastic differences in the behaviour of feline and bovine adrenal chromaffin cells. It might well be that the control by muscarinic receptors of such electrical properties considerably differ between cells from both animal species.

Calcium-dependent potassium channels and the muscarinic secretory response

DTC, apamin and TEA all increased the secretory responses to methacholine. In addition, DTC and apamin augmented markedly the Ca^{2+} signal generated by muscarinic stimulation. Since these agents are known to block various types of K^+ channels, it seems that we can safely conclude that such channels are involved in the regulation of the muscarinic secretory response in the cat adrenal chromaffin cell. The question is to define which subtype of K^+ channel might be the protagonist of such a modulatory role.

In principle, the most pronounced potentiating effects of the $[Ca^{2+}]_i$ signal were seen with apamin. This bee venom component is considered to be quite specific as a blocker of SK Ca^{2+}-dependent K^+ channels in sympathetic neurones (Nohmi & Kuba, 1984; Kawai & Watanabe, 1986) and chromaffin cells (Artalejo & Neher, 1991). As apamin, DTC blocks the after-hyperpolarization following an action potential, which is known to be due to activation of SK channels (Nohmi & Kuba, 1984; Blatz & Magleby, 1987). Though long depolarizing currents caused few spikes

in sympathetic neurones, greater repetitive firing was observed in the presence of apamin (Kawai & Watanabe, 1986). If this fact can be extrapolated to cat chromaffin cells, then the greater susceptibility of the methacholine secretory response to dihydropyridines, in the presence of DTC, might be explained by the recruitment of L-type Ca^{2+} channels during repetitive firing caused by blockade of SK channels.

Though a complete pharmacological dissection of Ca^{2+}-dependent K^+ currents is not feasible, it seems that DTC and apamin block better the SK channel while TEA does not block it. In contrast, TEA blocks BK channels but not SK channels. Blockade by TEA of BK channels is achieved with concentrations of 1 mM in chromaffin cells (Marty & Neher, 1985) and 1–5 mM in NG108-15 cells (Brown & Higashida, 1988) and sympathetic neurones (Smart, 1987). At these concentrations, TEA did not enhance the secretory responses to methacholine. The potentiation of methacholine secretion by higher concentrations of TEA might be associated with other effects of this ion unrelated to Ca^{2+}-dependent K^+ channels. Neither DTC nor apamin increased by themselves the basal levels of $[Ca^{2+}]_i$ or the basal rate of catecholamine secretion. In contrast, TEA enhanced it markedly. This increase has been attributed to direct depolarizing effects of TEA in cat chromaffin cells (Sorimachi *et al.* 1990) probably associated with direct nicotinic receptor stimulation. In fact, DTC blocked the TEA-evoked secretory response (not shown).

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