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VOLTAGE-DEPENDENT INACTIVATION OF CATECHOLAMINE SECRETION EVOKED BY BRIEF CALCIUM PULSES IN THE CAT ADRENAL MEDULLA

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SUMMARY

1. Inactivation by voltage changes of $^{45}Ca^{2+}$ uptake into and catecholamine release from cat adrenal glands perfused at a high rate (4 ml/min) at 37 °C with oxygenated Krebs–Tris solution has been studied. Experimental conditions were selected so that adrenal medullary chromaffin cells were depolarized for different time periods and with various K^+ concentrations in the absence of Ca^{2+}, prior to the application of 0·5 mM-Ca^{2+} for 10 s in the presence of 118 mM-K^+ to test the rate of secretion (the 'Ca^{2+} pulse').

2. Application of the Ca^{2+} pulse after perfusion with 5·9 mM-K^+ led to a 100-fold increase of the basal rate of secretion. However, if the Ca^{2+} pulse was preceded by a 10 min period of perfusion with 118 mM-K^+, the secretory response was decreased by over 80%.

3. Inactivation of secretion starts 10–30 s after high-K^+ perfusion and is completed 2–5 min thereafter. Inactivation is readily reversed by perfusing the glands with normal K^+-containing solution; the recovery phenomenon is also gradual and time-dependent, starting 30 s after repolarization and ending 300 s thereafter.

4. The rate of inactivation is much slower at 35 than at 118 mM-K^+, suggesting that the process is strongly dependent on voltage.

5. Like catecholamine release, Ca^{2+} uptake into adrenal medullary chromaffin cells is inactivated in a voltage-dependent manner. This, together with the fact that Cd^{2+} blocked secretion completely and inactivation was seen equally using Ca^{2+} or Ba^{2+} as secretagogues, suggests that inactivation of a certain class of voltage-dependent Ca^{2+} channels is responsible for the blockade of secretion. Such channels must be slowly inactivated by voltage and highly sensitive to dihydropyridines, since (+)PN200-110 (an L-type Ca^{2+} channel blocker) enhanced the rate of inactivation and (±)Bay K 8644 (an L-type Ca^{2+} channel activator) prevented it, indicating that they might belong to L-subtype Ca^{2+} channels.

6. The effects of (±)Bay K 8644 (100 nM) were seen on both the voltage and time dependence of inactivation. At a moderate depolarization (35 mM-K^+), the drug

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MS 7895

prevented inactivation and caused potentiation of secretion which developed gradually; at strong depolarizations (118 mM-K^+), Bay K 8644 prevented the time-dependent development of inactivation. (+)PN200-110 (30 nM) did not suddenly decrease catecholamine release at the earlier times of depolarization; what the drug did was to accelerate the normal rate of inactivation induced by depolarization. These observations strongly suggest that Bay K 8644 and (+)PN200-110 are mimicking a phenomenon which is taking place physiologically, and supports the idea that an endogenous dihydropyridine-like substance is regulating the rate of opening of Ca^{2+} channels in chromaffin cells.

INTRODUCTION

Attempts to correlate voltage-sensitive Ca^{2+} channel activities with the regulation of secretion are exemplified in various parallel studies of $^{45}Ca^{2+}$ fluxes and catecholamine release in chromaffin cells (Douglas & Poisner, 1962; Baker & Rink, 1975; Kilpatrick, Slepetis, Corcoran & Kirshner, 1982; Holz, Senter & Frye, 1982; Wada, Takara, Izumi, Kobayashi & Yanagihara, 1985; Artalejo, Bader, Aunis & García, 1986). The reverse is also true; analysis of the kinetics of secretion has been used to infer how Ca^{2+} channels might control the secretory process through changes in their kinetics. So, measuring catecholamine release, Baker and Rink suggested in 1975 that the Ca^{2+} channel that was mediating secretion from perfused bovine adrenal glands inactivated in the voltage-dependent manner.

Interpretation of these earlier studies is complicated in the light of later reports using more direct approaches demonstrating that major components of depolarization-evoked $^{45}Ca^{2+}$ uptake (Artalejo, García & Aunis, 1987) and Ca^{2+} currents (Hirning, Artalejo, Dahmer, Perlman & Fox, 1989) are inactivated in a Ca^{2+}- but not voltage-dependent manner. A further complication is the fact that to enter the cell interior during depolarization of bovine chromaffin cells, external Ca^{2+} ions might use more than one channel type. For instance, the 1,4-dihydropyridine derivative Ca^{2+} channel activator Bay K 8644 potentiates K^+-evoked Ca^{2+} uptake at low depolarizing levels (García, Sala, Reig, Viniegra, Frias, Fonteríz & Gandía, 1984), yet the blockers nitrendipine or nifedipine inhibit it only partially (Ballesta, Palmero, Hidalgo, Gutierrez, Reig, Viniegra & García, 1989; Rosario, Soria, Feuerstein & Pollard, 1989). Binding studies using [^{3}H](+)PN200-110 (Castillo, Fonteríz, López, Rosenheck & García, 1989), [^{3}H]nitrendipine (García *et al.* 1984; Ballesta *et al.* 1989) and ^{125}I-labelled ω-conotoxin (Ballesta *et al.* 1989) as ligands also suggest the presence of heterogeneous functional and binding sites associated with different Ca^{2+} channels. This idea has been recently strengthened with patch-clamp recordings of various types of Ca^{2+} currents in cultured ox chromaffin cells (Artalejo, Dahmer, Perlman & Fox, 1989; but see Fenwick, Marty & Neher, 1982; Hoshi, Rothlein & Smith, 1984; Hoshi & Smith, 1987).

In contrast to the bovine, the cat adrenal gland secretory response is highly sensitive to nanomolar concentrations of dihydropyridine Ca^{2+} channel activators (Montiel, Artalejo & García, 1984) and blockers (Gandía, López, Fonteríz, Artalejo & García, 1987). Its unique characteristics as a model to study correlations between the activities of Ca^{2+} channels and secretion are emphasized by the fact that other

catecholamine-storing cells such as the widely used bovine chromaffin cells (Ballesta *et al.* 1989; Rosario *et al.* 1989) or sympathetic neurones (Hirning, Fox, McCleskey, Olivera, Thayer, Miller & Tsien, 1988) are highly resistant to dihydropyridines. However, because the cat adrenal secretory response is fully blocked by various dihydropyridine derivatives in a voltage-dependent manner (Artalejo, López, Moro, Castillo, Pascual & García, 1988*b*; López, Moro, Castillo, Artalejo & García, 1989) it seems that a single type of Ca^{2+} channel, pharmacologically identified as the L-subtype (see review by Artalejo, López, Castillo, Moro & García, 1988*a*) dominates the control of secretion in the feline chromaffin cell. Because this type of Ca^{2+} channel is so closely associated with the control of secretion, and it is so easily manipulated pharmacologically, we felt that we could approach a study to its regulation by quantifying functional parameters (i.e. $^{45}Ca^{2+}$ uptake and catecholamine release) which will closely follow alterations of the rates of opening of Ca^{2+} channels induced by voltage changes, different divalent cations or dihydropyridine Ca^{2+} channel activators and blockers. In so doing, we should be able to make conclusions about the regulation by voltage and Ca^{2+} changes of the secretory process, in conditions (intact gland) more physiological than in isolated single cells, where the secretory process cannot be studied (see Discussion).

To achieve this, we used experimental protocols to study the rates of secretion that closely mimic those used to measure Ca^{2+} currents by voltage-clamping the membrane potential of bovine chromaffin cells, except for the unavoidable time scale difference. We feel that these protocols are original in the following aspects (see comments in the Results section):

(i) Catecholamine release was measured in the few-seconds range by using brief pulses (1–10 s) of low concentrations of Ca^{2+} or Ba^{2+} ions which are injected into the fluid stream perfusing the glands at very high flow rates. This avoids saturation of the secretory response by excess divalent cations, thereby allowing sharper discrimination of small changes in the rates of catecholamine release which can be quantified at different time periods of depolarization. With this procedure, no decline of the secretory response upon successive stimulations is seen in the same gland throughout the experiment.

(ii) Clamping the membrane potential of chromaffin cells chemically through the use of different concentrations of external K^+ in the absence of Ca^{2+} for various time periods. This allows the extrapolation of experimental protocols currently used to analyse the kinetics of Ca^{2+} currents (Hagiwara & Byerly, 1981) to the study of secretion; these protocols are known to cause inactivation of various types of Ca^{2+} channels in a voltage-, Ca^{2+}- and time-dependent manner.

(iii) Estimating Ca^{2+} uptake using $^{45}Ca^{2+}$ as radiotracer under various depolarizing conditions and short stimulation periods, to insure that changes seen in the rates of secretion certainly reflect changes in activities of Ca^{2+} channels.

(iv) Using dihydropyridine Ca^{2+} channel blockers and activators to modify the time- and voltage-dependent rates of inactivation of secretion evoked by Ca^{2+} pulses in glands depolarized for different time periods at various K^+ concentrations.

All these experimental approaches clearly differ from those of Baker & Rink (1975) and Schiavone & Kirpekar (1982) who did not use dihydropyridine drugs and did not establish the gradual time dependence of inactivation and reactivation of secretion in

the few-seconds range. Secretion in these conditions seems to be inactivated in a Ca^{2+}-independent manner. Rather, it inactivates and reactivates in a voltage- and time-dependent manner, suggesting that during depolarization of chromaffin cells, a biochemical process is slowly developing to inactivate and reactivate secretion under the prolonged voltage changes taking place during stressful situations. Such processes of inactivation and reactivation are closely mimicked by dihydropyridines; therefore, this can be taken as further functional evidence for the belief that a putative endogenous dihydropyridine-like substance is controlling the gating of such channels and, indirectly, the rate of secretion through a dihydropyridine receptor probably coupled to L-type Ca^{2+} channels.

METHODS

Cats (3–4 kg weight) were anaesthetized with sodium pentobarbitone, 40 mg/kg I.P. Both adrenal glands from each animal were isolated and prepared for their retrograde perfusion as previously described (García, Hernández, Horga & Sánchez-García, 1980). They were perfused at a rate of 4 ml/min, with Krebs–Tris solution at 37 °C and pH 7·4, continuously bubbled with pure O_2 and having the following composition (mM): NaCl, 144; KCl, 5·9; $MgCl_2$, 1·2; $CaCl_2$, 2·5; Tris, 10; and glucose, 11. Glands were initially perfused with this solution for 60 min to allow their equilibration with the novel *in vitro* conditions.

Experimental design: monitoring catecholamine release rates after application of brief calcium pulses

The experiments were designed to test how variable periods of depolarization of the *in situ* adrenal chromaffin cells, in the absence of external Ca^{2+} ions, affected the catecholamine secretory response evoked by 10 s pulses of Ca^{2+}. This was achieved as follows.

After 1 h in Krebs–Tris solution, the basic perfusion solution was a normal Krebs–Tris solution lacking Ca^{2+} and containing 5·9 mM-K^+; we will refer to it as 5·9 K^+/0 Ca^{2+} solution. These basal 'silent secretion conditions' were interrupted at regular intervals (usually 15–20 min) with stimulation test pulses to determine the rate of catecholamine release at any given time. The pulses consisted of switching for 10 s from 5·9 K^+/0 Ca^{2+} to a solution containing 118 mM-K^+ (NaCl was reduced equiosmotically) and 0·5 mM-Ca^{2+}; this solution will be referred to as 118 K^+/0·5 Ca^{2+} or 'Ca^{2+} pulse'. In these conditions, we explore the gland secretory rate from a preceding polarized situation of their chromaffin cells.

When the effects of preceding depolarization periods of variable lengths were explored, each 20 min period in 5·9 K^+/0 Ca^{2+} is reduced by the same amount of time as the gland is depolarized prior to the Ca^{2+} pulse. For instance, if the effects of a 10 min depolarization with 118 K^+/0 Ca^{2+} on secretion are being explored, the 20 min period preceding the Ca^{2+} pulse is divided in a 10 min sub-period of perfusion with 5·9 K^+/0 Ca^{2+} plus another with 118 K^+/0 Ca^{2+} (see protocol at the top of Fig. 1). With this protocol, and since the secretory response is maintained healthy for several hours, one can explore in the same gland how the length of the depolarization period affects the rate of secretion (time dependence of inactivation); various 20 min periods in 5·9 K^+/0 Ca^{2+} are divided sequentially into sub-periods of perfusion with 5·9 K^+/0 Ca^{2+} plus different times (0, 10, 20, 30, 60, 120, 300, 600 s) of perfusion with 118 K^+/0 Ca^{2+}.

The voltage dependence of inactivation is studied by perfusion of the glands with low (35 K^+/0 Ca^{2+}) or high (118 K^+/0 Ca^{2+}) depolarizing solutions for different time periods before each Ca^{2+} pulse. Since glands are perfused always with Ca^{2+}-free solutions (except for the brief secretory test pulses), a possible Ca^{2+}-dependent component in the voltage-dependent inactivation of secretion can be ruled out.

Because inactivation of secretion is reversible, it was possible to design an experiment in order to study whether recovery from inactivation was instantaneous or time-dependent. The glands were perfused several times with 118 K^+/0 Ca^{2+} for 10 min to provoke a fixed degree of inactivation. After this period, perfusion with a repolarizing solution (5·9 K^+/0 Ca^{2+}) was

intercalated between the depolarization and the Ca^{2+} pulse; this 'repolarizing' period was varied between 0 and 600 s before the secretory test pulse was applied (see protocol at the top of Fig. 4).

When used, dihydropyridine derivatives were diluted in the appropriate perfusing solution from concentrated solutions in ethanol; dilutions and experiments were performed under sodium light.

Assay of total catecholamines present in perfusate fluid samples

The basal rate of catecholamine release ('silent' period, in Ca^{2+}-free solutions) was monitored immediately before the Ca^{2+} pulse by collecting a 30 s sample in 5·9 K^+/0 Ca^{2+}, 35 K^+/0 Ca^{2+} or 118 K^+/0 Ca^{2+} solutions, depending on the experimental protocol. The catecholamine content in these conditions was almost undetectable fluorometrically. Catecholamines released during each Ca^{2+} pulse were collected in two additional 30 s samples; the first included the 10 s in 118 K^+/0·5 Ca^{2+} plus 20 s back in 5·9 K^+/0 Ca^{2+} plus an additional 30 s sample in this last solution, to wash out completely catecholamines released but still present in extracellular spaces.

These fluid samples were collected in acidified (0·05 M-perchloric acid, final) chilled tubes and fluorometrically assayed, directly, in 1 ml aliquots using the iodine oxidation procedure described by Shellenberger & Gordon (1971) to develop the total catecholamine fluorescence. Fluorescence units were converted into micrograms of total catecholamines through the use of appropriate standards of the pure amines. Data are usually expressed as micrograms per Ca^{2+} pulse with their S.E.M. They reflect the sum of catecholamines present in the two stimulation samples after subtracting from them the basal catecholamine rate (before the Ca^{2+} pulse).

Calcium uptake into adrenal medullary tissues

The rate of Ca^{2+} uptake into adrenal medullary chromaffin cells was determined by incorporating tracer amounts of $^{45}Ca^{2+}$ (16 μCi/ml, 25·3 mCi/mg, Amersham) into the solution to test the secretory rate (118 K^+/0·5 Ca^{2+}). Since the adrenal medullary tissue had to be dissected out from the cortex in these experiments, two Ca^{2+} pulses were given before the $^{45}Ca^{2+}$ test pulse, in order to see, in the same glands, whether inactivation of secretion took place after a 10 min period of depolarization with 118 K^+/0 Ca^{2+}. A 2 min wash-out period with 5·9 K^+/0 Ca^{2+} or 1·2 K^+/0 Ca^{2+} solutions containing 2 mM-$LaCl_3$ and 2 mM-EGTA followed; this served to wash out extracellular, unbound $^{45}Ca^{2+}$. After this, the adrenal medulla was carefully dissected from the cortex and homogenized in 2 ml of 10% trichloroacetic acid. Aliquots (100 μl) of this homogenate were added to 4 ml scintillation fluid (Insta-Gel II, Packard) and counted in a Packard Tri-Carb 1500 liquid scintillation analyser. Proteins were estimated in aliquots of the homogenate after neutralization with NaOH, using bovine serum albumin as standard (Lowry, Rosebrough, Farr & Randall, 1957).

Statistical analyses of means were performed with the computer program MICROSTAT (Copyright Ecosoft, Inc., 1985). Level of significance between means was taken at $P < 0.05$.

RESULTS

The central observation in this section is a near abolition of catecholamine release responses when cat adrenal glands are challanged with brief Ca^{2+} pulses (usually 0·5 mM for 10 s, in the presence of 118 mM-K^+), given after different periods of perfusion with Ca^{2+}-free solutions containing high K^+. For the sake of clarity, we will call depolarization that period preceding any Ca^{2+} pulse to challenge the adrenal medullary chromaffin cells to secrete catecholamines. It has been demonstrated that in rat (Ishikawa & Kanno, 1978) and ox adrenal glands (Baker & Rink, 1975), high K^+ concentrations cause a sustained depolarization of their chromaffin cells.

Catecholamine release evoked by calcium pulses from glands perfused with solutions containing normal or high potassium but no calcium

Figure 1 shows the central finding of this study. When glands are perfused for 10 min with a solution lacking Ca^{2+} but containing the rest of the normal components of the basic Krebs–Tris solution used here, and then K^+ and Ca^{2+} are increased for

10 s to 118 and 0·5 mM respectively (from now onwards, we will refer to this secretory challenge as the Ca^{2+} pulse), catecholamine release suddenly rises from about 0·02 to $2·3 \pm 0·3$ μg. There is, therefore, a 100-fold increase in the basal rate of secretion. However, if the same glands are perfused also for 10 min, prior to the Ca^{2+} pulse, with

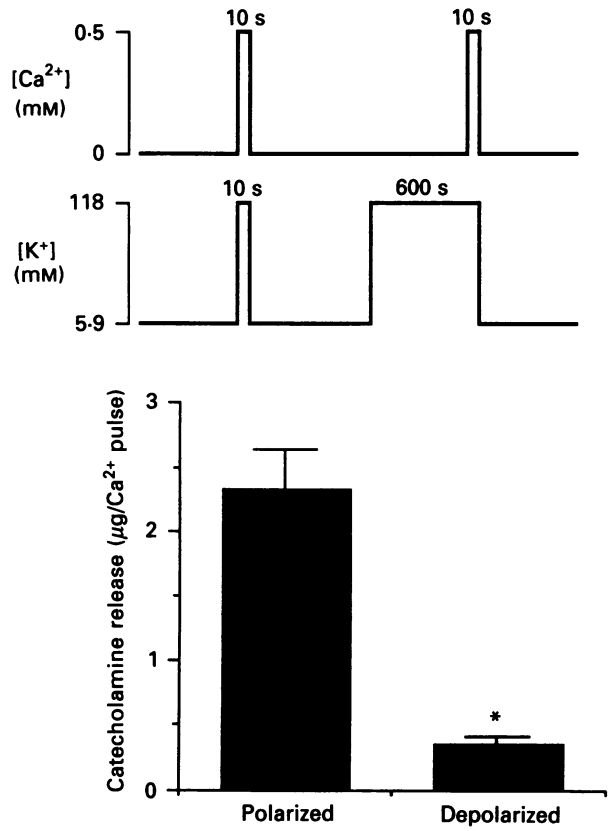

Fig. 1. Suppression of catecholamine release from depolarized chromaffin cells. The experimental protocol appears at the top of the figure. First, glands were perfused with a Krebs–Tris–0 Ca^{2+} solution containing 5·9 mM-K^+ (polarized) for 10 min (5·9 K^+/0 Ca^{2+}); then, catecholamine release was evoked by switching this solution to another containing 118 mM-K^+ and 0·5 mM–Ca^{2+} for 10 s (118 K^+/0·5 Ca^{2+}). After this, glands were perfused for 10 min with 5·9 K^+/0 Ca^{2+} and ten additional minutes with 118 K^+/0 Ca^{2+} (depolarized); then, the 10 s Ca^{2+} pulse was applied as above in the presence of 118 mM-K^+. Catecholamines present in samples of perfusion fluid collected during the 10 s Ca^{2+} pulse and 50 s thereafter in 5·9 K^+/0 Ca^{2+} are expressed in μg/Ca^{2+} pulse (ordinate). Data are means + S.E.M. of twenty experiments. $*P < 0·01$.

a Ca^{2+}-free solution enriched in K^+ (118 K^+/0 Ca^{2+}), and then the Ca^{2+} pulse is applied, secretion goes down to $0·35 \pm 0·06$ μg. Depolarization, then, blocked the secretory response to Ca^{2+} by over 80% ($n = 20$).

Blockade of secretion by depolarization is a reversible phenomenon which can be repeated several times in the same gland. Figure 2 shows the rates of secretion

obtained by alternating periods of perfusion of the glands with solutions lacking Ca^{2+} and containing normal (5·9 mM) or high (118 mM) K^+. In addition, this figure shows the catecholamines recovered in each of the three individual 30 s samples (one pre-pulse sample and two pulse samples) of perfusion fluid collected in each Ca^{2+} pulse

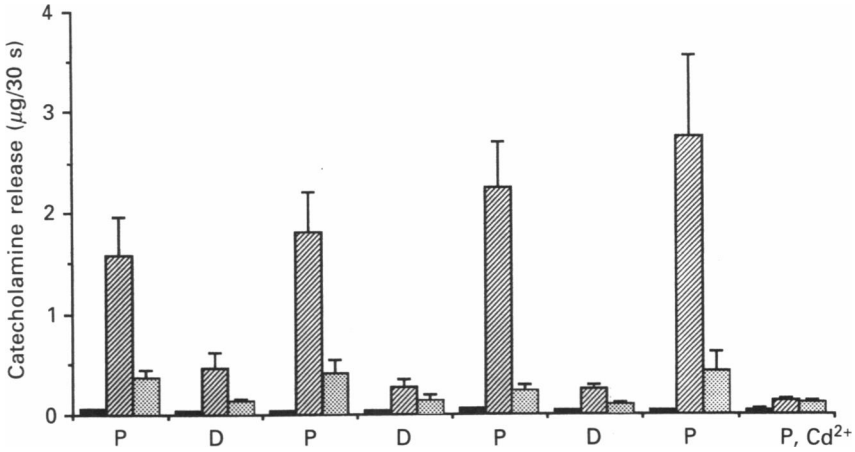

Fig. 2. Reversibility of the suppression of catecholamine release induced by depolarization and the effect of Cd^{2+} ions on secretion. The protocol was similar to the one described in Fig. 1, but here three pairs of Ca^{2+} pulses (0·5 mM for 10 s) were applied sequentially to the same gland after 10 min periods of perfusion with 5·9 K^+/0 Ca^{2+} (P, polarized) or 118 K^+/0 Ca^{2+} (D, depolarized for 10 min). At the end of the experiments, two more Ca^{2+} pulses were applied after 10 min perfusion with 5·9 K^+/0 Ca^{2+}, one in the absence and the last in the presence of 2 mM-Cd^{2+}. Cd^{2+} was present since 10 min before and during the 10 s pulse with 118 K^+/0 Ca^{2+}. Data are means ± S.E.M. of four experiments. ■, basal; ▨, sample 1; ▩, sample 2.

(see Methods). The alternating 10 min periods of pre-perfusion with 5·9 K^+/0 Ca^{2+} or 118 K^+/0 Ca^{2+} are accompanied also by high (polarized) or low (depolarized) levels of catecholamine release. Most of the catecholamines (over 80%) are recovered during the first 30 s sample collected after the Ca^{2+} pulse. Blockade and recovery of secretion were seen at least 3 times in the same gland using this protocol.

It is worth noting that each depolarization period is followed by an enhanced secretion after the non-depolarized period, as if the previous blockade of secretion could cause a facilitation during recovery. An additional feature in this experiment is the fact that Cd^{2+} ions (2 mM) completely blocked secretion in glands perfused with 5·9 K^+/0 Ca^{2+}, suggesting that Ca^{2+} channels are mediating the 'normal' secretory response in polarized conditions.

Time dependence of the inactivation of secretion produced by perfusion with high potassium concentrations

The next question we posed was whether depolarization-evoked inactivation of catecholamine release was a gradual, time-dependent or an all-or-none phenomenon. Since we already knew that such a process was reversible, and that several alternating cycles of inactivation and reactivation could be demonstrated in the same

gland, we could design and perform an experiment to test this question. Figure 3 shows that inactivation of secretion increases with the time of depolarization: 50% inactivation occurs after 30 s. Inactivation continues gradually to stabilize at about 10–15% of the initial secretion rate after 5 min depolarization. Inactivation was

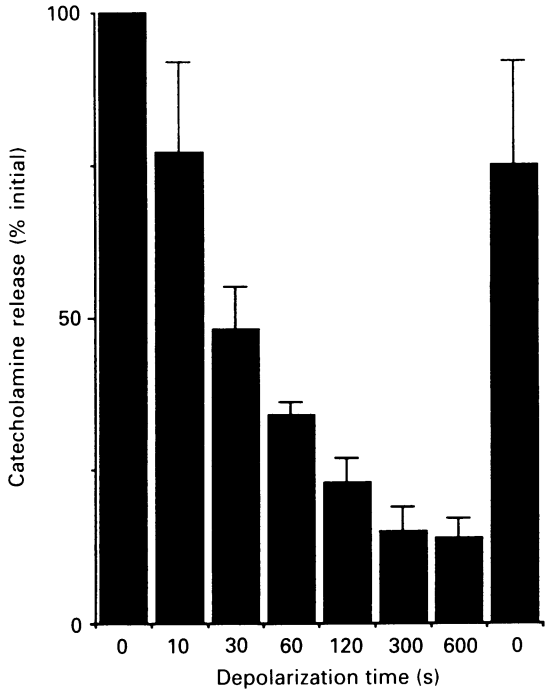

Fig. 3. The effects of increasing gradually the time of perfusion with 118 K^+/0 Ca^{2+} on catecholamine release evoked by Ca^{2+} reintroduction (0·5 mM for 10 s after each of the depolarization times shown in the abscissa). Each of the three experiments were performed in the same adrenal gland as follows: the initial rate of secretion (100%) was obtained after perfusion with 5·9 K^+/0 Ca^{2+} for 10 min and then 10 s with 118 K^+/0·5 Ca^{2+} (test Ca^{2+} pulse). Then, at 15 min intervals, the time of perfusion with 5·9 K^+/0 Ca^{2+} was gradually decreased and substituted by equivalent periods (10–600 s) of perfusion with 118 K^+/0 Ca^{2+} solutions (depolarization times shown on the abscissa). At the end of the experiment, the perfusion period was again entirely performed in 5·9 K^+/0 Ca^{2+} (zero-time depolarization). Catecholamine released in each test Ca^{2+} pulse was normalized to 100% (secretion initially obtained without depolarization). Data are means+S.E.M. of three experiments.

completely reversed after perfusion of the glands for 10 min with the solution containing normal K^+ (0 s depolarization in the right part of Fig. 3).

Recovery from inactivation of secretion

If inactivation were a time-dependent process, it would be likely that the recovery was also time-dependent. This was tested in the same adrenal gland by fixing the time of perfusion with 118 K^+/0 Ca^{2+} at 10 min, which is the time when maximum inactivation takes place, and varying the period of repolarization (perfusion with 5·9 K^+/0 Ca^{2+}) from 0 to 600 s. Figure 4 shows that secretion gradually recovered

with the time of repolarization. Half-recovery was obtained after 30–60 s of repolarization, similar to the depolarization time required for half-inactivation of secretion (Fig. 3). It seems as if the mechanism underlying both processes were similar but opposite, as a mirror image.

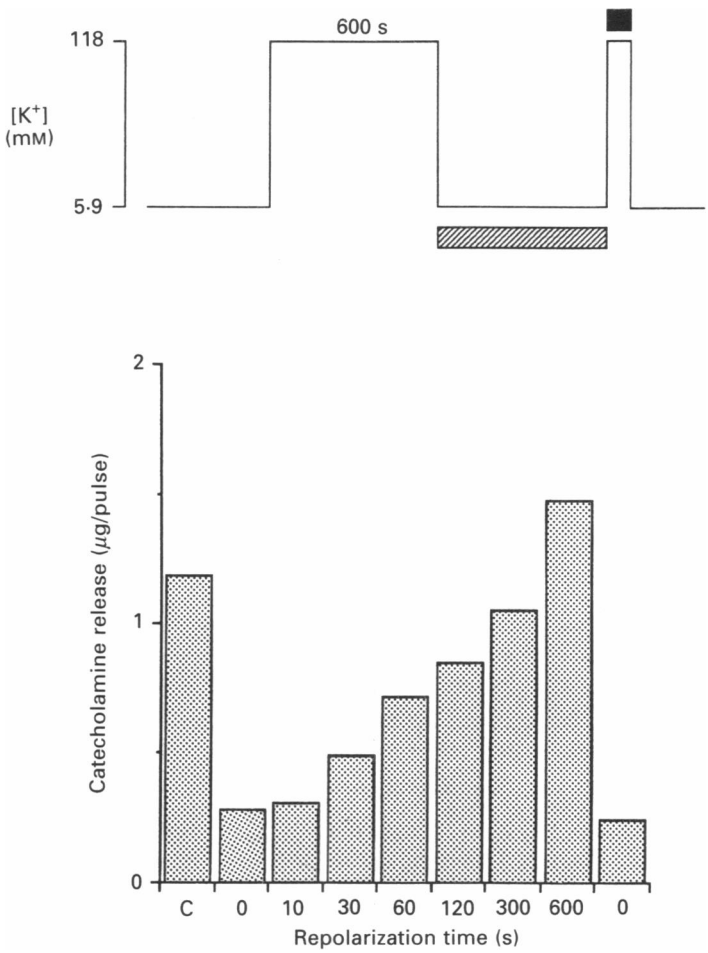

Fig. 4. Time dependence of the reactivation of secretion after its blockade by depolarization. After testing the control secretory rate (first column on the left) in resting conditions (10 s pulse with 118 K^+/0 Ca^{2+}), time-dependent recovery from inactivation was explored as follows: the gland was perfused for 10 min with 118 K^+/0 Ca^{2+}, seven times at 20 min intervals; each time, a period of repolarization (0–600 s, ▨) followed before the Ca^{2+} test pulse (0·5 mM for 10 s, ■) was applied. The last column to the right shows how, after recovery, secretion inactivates again when no repolarization follows the perfusion period with 118 K^+/0 Ca^{2+}. Data are from a single experiment; in a second experiment, essentially the same results were obtained.

Inactivation of secretion is equally seen using calcium or barium as secretagogues

Barium is a powerful secretagogue in the cat adrenal gland (Douglas & Rubin, 1964); since it permeates, even better than Ca^{2+}, certain channels in excitable cells (Hagiwara & Byerly, 1981; Kostyuk, 1989) we suspected that if voltage (but not

Ca^{2+}) was responsible for the inactivation of secretion, Ba^{2+} would provide the same degree of inactivation of secretion as Ca^{2+}.

To test this, two glands from the same cat were subjected to an experimental protocol very similar to that described above to assess the time dependence of

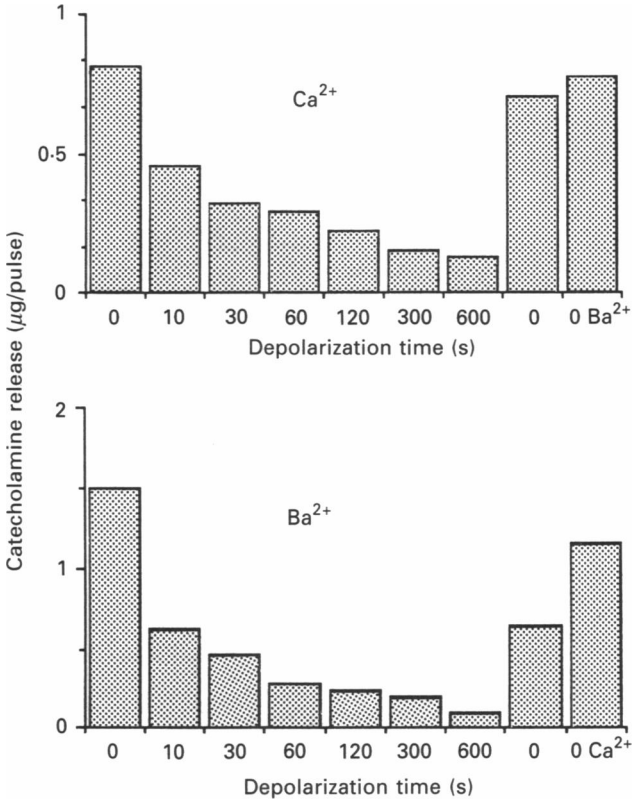

Fig. 5. Catecholamine release evoked by Ca^{2+} (top) or Ba^{2+} (bottom) pulses (0·5 mM for 10 s in the presence of 118 mM-K^+) from glands perfused for the time periods shown on the abscissae with a depolarizing solution (118 K^+/0 Ca^{2+} or 118 K^+/0 Ba^{2+}). The last two pulses were given, without a depolarization period, with Ca^{2+} and Ba^{2+} (top) or Ba^{2+} and Ca^{2+} (bottom), respectively. The protocol is very similar to that described in Fig. 3. except that here two glands from the same cat were used to test the secretory response to Ca^{2+} and Ba^{2+}. Data are from a single paired experiment; in a second experiment, similar results were obtained.

secretion inactivation by depolarization. Figure 5 (top) shows data which are very similar to those collected in Fig. 3; using Ca^{2+} (0·5 mM for 10 s) as a test pulse for secretion, a gradual inactivation is observed as the pre-perfusion time with 118 K^+/0 Ca^{2+} increases. Using Ba^{2+} as secretagogue (bottom of Fig. 5), a similar inactivation profile is observed; here, the starting secretion level was higher (about 1·5 μg) than with Ca^{2+} (about 0·8 μg), corroborating the earlier observation of Douglas & Rubin (1964) that Ba^{2+} is more potent than Ca^{2+} in evoking secretion.

Recovery from inactivation was tested at the end of this experiment by crossing the divalent cation pulses between the two glands. After perfusing the glands for

20 min with 5·9 K^+/0 Ca^{2+} solutions, secretory responses to Ca^{2+} and Ba^{2+} were greatly restored in both glands (last two columns in the right part of Fig. 5).

In other experiments, the possibility of seeing the inactivation of secretion under more severe and variable conditions was tested. Pairs of glands were continuously

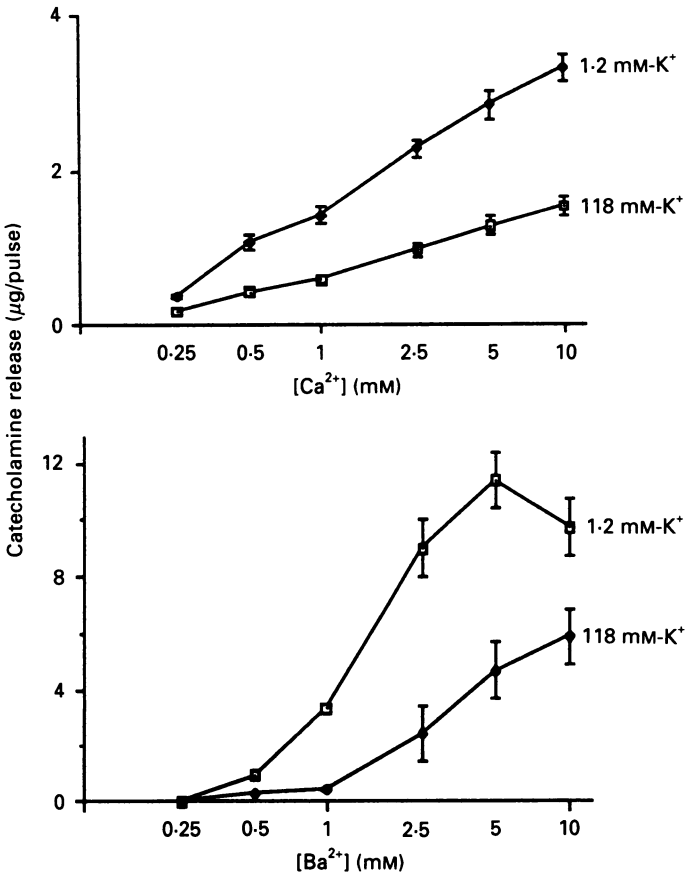

Fig. 6. Catecholamine release evoked by 1 s pulses of increasing concentrations of Ca^{2+} (top) or Ba^{2+} (bottom) given intermittently to glands continuously perfused with solutions lacking Ca^{2+} and Ba^{2+} and containing 1·2 or 118 mM-K^+ as indicated. At 20 min intervals, 0·6 ml of the secretory test solution (118 mM-K^+ containing each of the concentrations of the divalent cation shown on the abscissae) were quickly injected into the fluid stream perfusing the gland through a three-stop-cock valve placed at the exit of the adrenal vein. The approximate time of injection and wash-out of the fluid was 1 s, though this cannot be known with precision. Data are expressed in micrograms of catecholamine released per test pulse (ordinates) and are means ± S.E.M. of seven glands for Ca^{2+} and four glands for Ba^{2+}.

perfused with solutions lacking Ca^{2+} or Ba^{2+} and containing low (1·2 mM) or high (118 mM) K^+ concentrations. Secretion was evoked by giving short 1 s secretory pulses with solutions containing 118 mM-K^+ and increasing concentrations of Ca^{2+} or Ba^{2+} (Fig. 6, top and bottom, respectively). This quick stimulation was selected in order to avoid saturation by excess divalent cations of the secretory mechanism,

thereby allowing a closer relationship to establish between the rate of secretion and the concentration of the divalent cation present in the test-pulse solutions.

Catecholamine release was proportional to the concentration of divalent cations present in the solutions to test the rate of secretion,

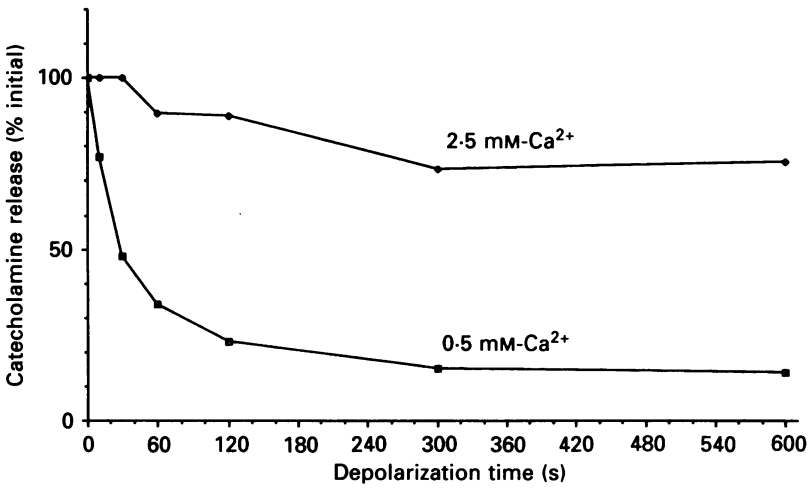

Fig. 7. The protocol of this experiment is similar to that described in Fig. 3. In one gland, the secretory test solution contained 0·5 mM-Ca^{2+} and in the contralateral gland from the same cat, 2·5 mM-Ca^{2+}, both in the presence of 118 mM-K^+. Each Ca^{2+} test pulse was applied after different times of perfusion with a solution enriched with K^+ (118 mM) and deprived of Ca^{2+} (118 K^+/0 Ca^{2+}). The secretory rate was normalized in the absence of depolarization (ordinate). Data are from a single paired experiment; in a second experiment, similar results were obtained.

from 0·25 to 10 mM. Secretion was considerably higher with Ba^{2+}, and tended to reach a plateau at concentrations lower than with Ca^{2+}. Secretion was particularly diminished in depolarized glands at the lower concentrations of divalent cations. For instance, catecholamine release at 1 mM-Ba^{2+} was practically non-existent in 118 mM-K^+ but amounted to 3 μg in the gland perfused with 1·2 mM-K^+. This suggests that low concentrations of divalent cations must be used if one wishes to see inactivation of secretion by high K^+. If, as we believe, this inactivation of catecholamine release is due to inactivation of a certain subtype of Ca^{2+} channel (see later) this means that even if a large percentage of such channels are inactivated, an effect on secretion will be depicted more easily at the concentrations of divalent cations not saturating the secretory machinery. This is obvious since the rate of secretion should be proportional not only to the number of Ca^{2+} channels activated in each moment, but also to the gradient of $[Ca^{2+}]_o/[Ca^{2+}]_i$.

The reason for the uncovering of a much greater degree of inactivation of secretion, in comparison with earlier reports

By using 2·5 instead of 0·5 mM-Ca^{2+} to test the secretory rate after different time periods of perfusion with 118 K^+/0 Ca^{2+}, we could probably never have uncovered the clear time-dependent inactivation shown in Fig. 3. That this is so is proven in the

experiment summarized in Fig. 7. An experimental protocol similar to that shown in Fig. 3 was performed; in one gland the secretory test solution contained 0·5 mM-Ca^{2+} and in the contralateral gland 2·5 mM. Secretion was normalized in both cases to the initial rate obtained in the absence of perfusion with 118 K^+/0 Ca^{2+}. It is clear that

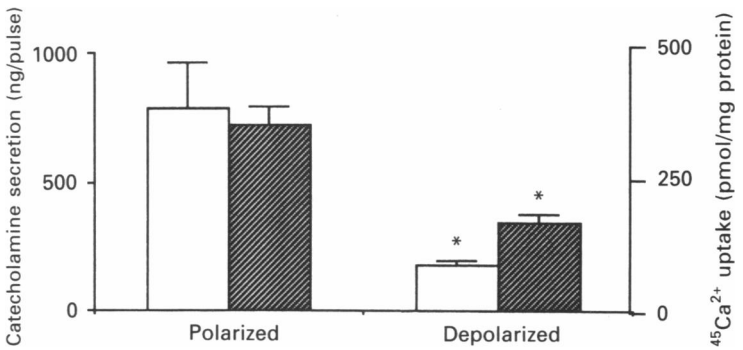

Fig. 8. Ca^{2+} uptake into (□), and catecholamine release from (▨), intact adrenal medullary tissues under polarizing or depolarizing conditions. The experiments followed a protocol similar to that of Fig. 2. First, secretory rates were tested alternately after two periods of polarizing (perfusion for 10 min with a 0 Ca^{2+} solution containing 5·9 mM-K^+) or depolarizing conditions (perfusion for 10 min with a 0 Ca^{2+} solution containing 118 mM-K^+). This insured that the glands behaved in the expected way: blockade of secretion by depolarization. Then, the 5th stimulus was applied with a solution containing $^{45}Ca^{2+}$ (16 μCi/ml) plus Ca^{2+} (0·5 mM for 10 s); in one gland this pulse was given after 10 min perfusion with 5·9 K^+/0 Ca^{2+} (polarized), and in the contralateral gland from the same animal after perfusion with 118 K^+/0 Ca^{2+} (depolarized). After washing the adrenal glands with a Ca^{2+}-free solution, the medullae were carefully dissected from the cortex and processed to determine their radioactive Ca^{2+} contents as described in Methods. Data are means ± S.E.M. of three paired experiments. * $P < 0.01$, compared with polarized glands.

while the inactivation was very pronounced in 0·5 mM-Ca^{2+}, it was very mild in 2·5 mM-Ca^{2+}.

We believe that this fact might have hampered the recognition of higher rates of inactivation of secretion in previous reports from our (Sala, Fonteríz, Borges & García, 1986) and other laboratories (Baker & Rink, 1975; Schiavone & Kirpekar, 1982). In fact, Baker & Rink (1975) recognized the difficulties in quantifying inactivation of secretion with their protocols by stating the following: 'quantitative comparison of the secretory responses of perfused glands was complicated by variation between glands in the size of responses and the progressive decline of the response of any one gland to successive stimulation throughout the course of an experiment'. Reintroducing 3·6 mM-Ca^{2+} in the presence of 72 mM-K^+ for 90 s, they obtained about 36% blockade of secretion under depolarizing conditions. A similar low degree of blockade by depolarization was observed by Schiavone & Kirpekar (1982) in perfused cat adrenal glands using 140 mM-K^+ and 2·5 mM-Ca^{2+} for 120 s. It is clear that these conditions were unable to unmask a higher degree of inactivation by depolarization of the secretory response. In our present experimental conditions, inactivation of secretion was much more pronounced than in those earlier studies; therefore, a kinetic analysis, and the study of the time dependence of inactivation

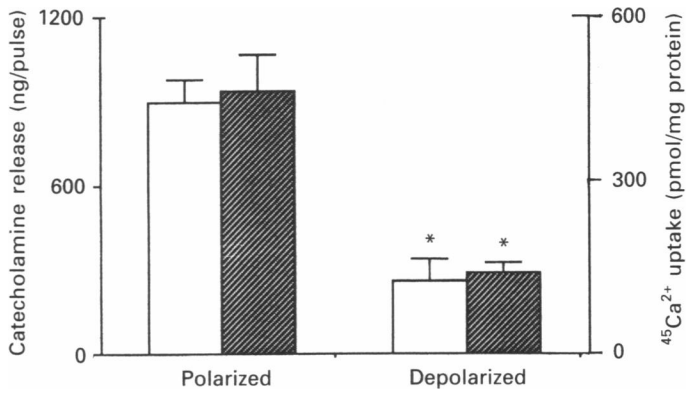

Fig. 9. Ca^{2+} uptake into (□), and catecholamine release from (▨), intact adrenal medullary tissues under polarizing or depolarizing conditions. The experiments were similar to those shown in Fig. 8, except for the following: polarized glands were perfused, before the Ca^{2+} pulse, with a Ca^{2+}-free solution containing 1·2 instead of 5·9 mM-K^+, and the depolarized glands with 118 K^+/0 Ca^{2+} for 20 min instead of 10 min. Data are means ± S.E.M. of eight experiments. $*P < 0.01$.

and recovery could be performed safely in the cat adrenal gland only under our present experimental conditions.

Inactivation of calcium uptake parallels inactivation of secretion

At this point in the investigation, a crucial question was whether Ca^{2+} channels were involved in the inactivation by depolarization of catecholamine release. The only way of answering it in the whole gland and under the same experimental conditions for secretion was by studying $^{45}Ca^{2+}$ uptake into chromaffin cells present in the intact adrenal medullary tissue. Two experimental designs were performed in order to test this hypothesis.

Results of the first protocol are summarized in Fig. 8. Three pairs of Ca^{2+} pulses were sequentially given to both adrenal glands from the same animal much in the same way to those seen in Fig. 2. Although in these experiments the inactivation of secretion by depolarization was lower (about 60%) than in those of Figs 1 and 2, it is worth noting that both inactivation of Ca^{2+} uptake and secretion run in parallel. Though they were paired experiments (polarized and depolarized glands from the same animals), data varied quite a lot. This was the reason for normalizing them, ascribing the 100% value to the polarized glands. Depolarization caused a 83 ± 6% decrease of $^{45}Ca^{2+}$ uptake, when compared to polarized glands.

The second protocol only differed in the solution perfusing the polarized glands, which instead of having 5·9 mM-K^+ contained only 1·2 mM-K^+. The depolarizing solution had the usual 118 mM-K^+ and no Ca^{2+}. Figure 9 shows the results. Blockade of Ca^{2+} uptake and catecholamine release were parallel and substantially higher (above 80%) than in the previous protocol.

Inactivation of secretion after perfusion with moderate and high concentrations of potassium

If, as hypothesized, inactivation of catecholamine release is voltage dependent, then the rate of inactivation with the time of depolarization would be slower in 35

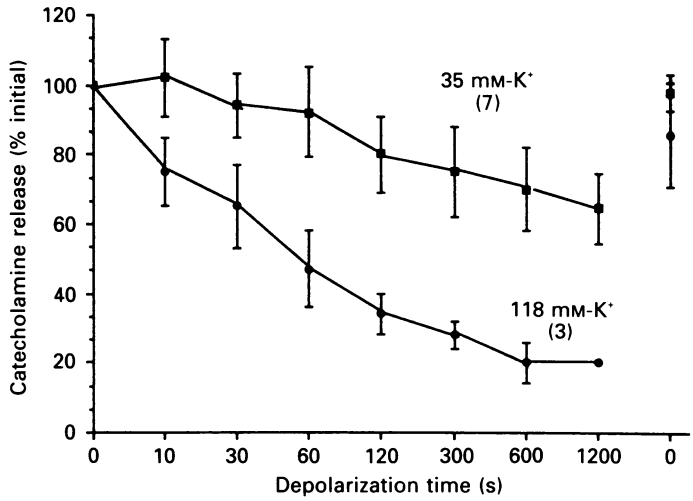

Fig. 10. Time dependence of inactivation of catecholamine release from glands perfused with Ca^{2+}-deprived solution containing 35 or 118 mM-K^+. The experimental protocol was similar to that described in Fig. 3; the difference resides only in the K^+ concentration of the solutions used to depolarize the glands for different time periods (abscissa). Note that after each perfusion period either with 35 or 118 mM-K^+, the secretory test solution contained always 118 mM-K^+ and 0·5 mM-Ca^{2+}, and was applied for 10 s, as usual. Data are normalized to the percentage of initial secretion from resting glands; they are means ± S.E.M. of the number of experiments shown in parentheses.

than in 118 mM-K^+. This certainly happened in the seven experiments shown in Fig. 10, where even after 20 min of perfusion with a 35 K^+/0 Ca^{2+} solution, only a 40% decrease in the rate of secretion evoked by the secretory test pulse (118 mM-K^+ plus 0·5 mM-Ca^{2+} during 10 s) was seen. The secretory response was decreased by over 50% when using 118 mM-K^+ during the first 60 s of perfusion; in contrast, it remained unaffected with 35 mM-K^+. The two last points on the right of Fig. 10 represent the secretion obtained without a depolarization period; note that the recovery is complete in both the glands perfused with 35 mM-K^+ and those perfused with 118 mM-K^+.

Effects of dihydropyridine calcium channel activator Bay K 8644 and blocker (+)PN200-110 on the time-dependent inactivation of secretion produced by depolarization

(±)Bay K 8644 is known to potentiate drastically K^+- and Ca^{2+}-evoked catecholamine release from perfused cat adrenal glands (García *et al.* 1984; Montiel *et al.* 1984); we also know that such potentiation is seen better at moderate K^+

concentrations (Ladona, Aunis, Gandía & García, 1987). On the other hand, (+)PN200-110 (isradipine) happens to be the most potent dihydropyridine derivative blocker of such a secretory response (Gandía *et al.* 1987). Therefore, in searching for possible mechanisms for the voltage-dependent inactivation of

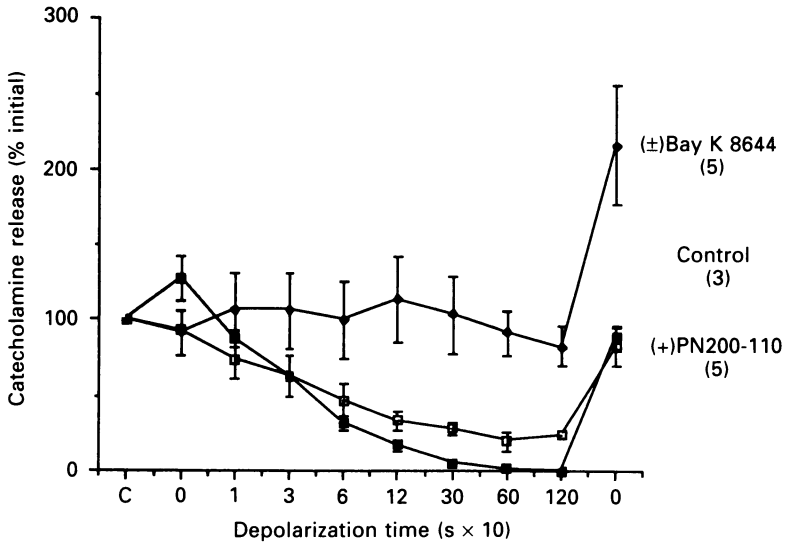

Fig. 11. Effects of (+)PN200-110 and (±)Bay K 8644 on the time-dependent inactivation of catecholamine release produced by a large depolarization. The control glands were manipulated in the same way as those in Fig. 3; at 15 min intervals, perfusion of the glands with a high-K^+ solution (118 mM) in the absence of Ca^{2+} was performed for different time periods (0–1200 s, abscissa). Then, after each depolarization period, the secretory test pulse was applied (0·5 mM-Ca^{2+} plus 118 mM-K^+ for 10 s). At the end of the experiment, recovery from inactivation was tested in resting conditions (last point in each curve). Glands treated with dihydropyridines were perfused with (+)PN200-110 (3×10^{-8} M) or with (±)Bay K 8644 (10^{-7} M) after the 0 s depolarization up to the end of the experiments; the other manipulations were identical to control glands. The experiments were performed in parallel (two glands from the same animal) in the case of (+)PN200-110- and (±)Bay K 8644-treated glands; controls were from different animals. Data, normalized to the initial rate of secretion (C = polarized gland, 100% on the ordinate), are means ± S.E.M. of the number of experiments shown in parentheses.

secretion, it was desirable to study the possible neutralizing (Bay K 8644) or additive ((+)PN200-110) effects of these drugs on the kinetics of this inactivation process.

Figure 11 shows that the rate of inactivation of secretion evoked by perfusion of the glands with 118 K^+/0 Ca^{2+} is greatly modified by (+)PN200-110 and (±)Bay K 8644. In the presence of (+)PN200-110 (30 nM), the rate of inactivation of secretion was accelerated, especially after the 30 s period of depolarization. An interesting feature in this experiment is the complete abolition of secretion in the presence of (+)PN200-110. Although depolarization in the absence of (+)PN200-110 causes over 80% inhibition of catecholamine release by itself, secretion was not abolished even after 20 min perfusion with 118 mM-K^+. A second feature is the full recovery of the secretory response in the presence of (+)PN200-110 when the perfusion solution was switched from 118 to 5·9 mM-K^+. This corroborates the idea

that the blocking effects of dihydropyridines are voltage dependent in the cat adrenal gland (Artalejo *et al.* 1988*b*; López *et al.* 1989).

The accelerating effects of (+)PN200-110 on the rate of inactivation were seen much better when 35 mM-K^+ was used during the depolarization period (Fig. 12).

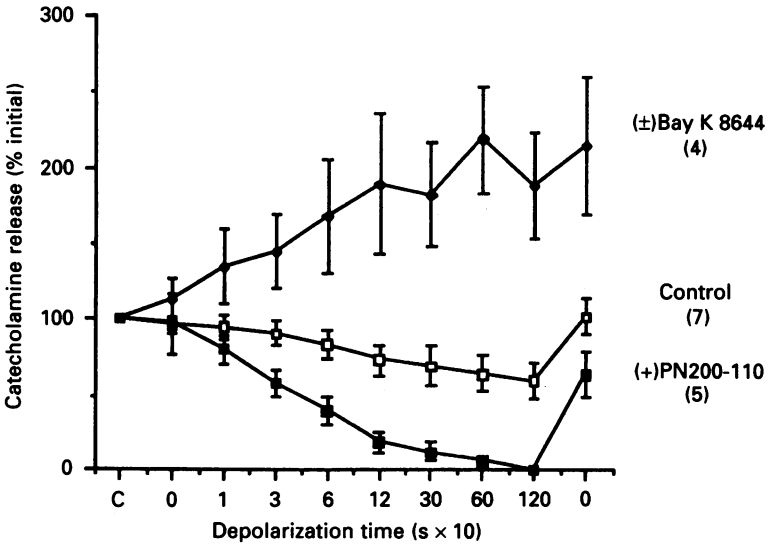

Fig. 12. Effects of (+)PN200-110 and (±)Bay K 8644 on the time-dependent inactivation of catecholamine release produced by a moderate depolarization. The experiment was similar to that shown in Fig. 11, except for the fact that the high-K^+ solution used to depolarize the glands contained a lower concentration of this cation (35 mM). Data, normalized to the initial rate of secretion (C = no depolarization, 100% on the ordinate), are means ± S.E.M. of the number of experiments shown in parentheses.

The mild inactivation seen in the absence of (+)PN200-110 is drastically accelerated in its presence; the dihydropyridine abolished secretion after 5 min perfusion with 35 K^+/0 Ca^{2+}. It is interesting to note that the combined effects of 35 mM-K^+ plus (+)PN200-110 were similar to the inactivation seen with 118 mM-K^+ as if K^+ and (+)PN200-110 were acting through a similar mechanism. Clear addition of effects of the two components of inactivation (voltage- and dihydropyridine-induced) is only seen at the lower K^+ concentration.

The effects of (±)Bay K 8644 were also striking. In 118 K^+/0 Ca^{2+}, (±)Bay K 8644 (100 nM) prevented the gradual, time-dependent inactivation of secretion, while in 35 K^+/0 Ca^{2+} the drug caused a gradual, time-dependent increase of the secretory response which started to stabilize after 5 min of perfusion with 35 K^+/0 Ca^{2+}. Though the progressive development of the effects of (±)Bay K 8644 is coincident with the gradual effects of (+)PN200-110, it is worth calling attention to the size of the secretory response on repolarizing the glands treated with (±)Bay K 8644 at the end of the experiment. In 118 K^+/0 Ca^{2+} a large potentiation of secretion was seen (more than 200% of the initial rate) while in 35 K^+/0 Ca^{2+} the rate of secretion was similar, with or without depolarization. This observation suggests two ideas: one, that the effects of (±)Bay K 8644 are voltage-independent,

the opposite to (+)PN200-110; and the other, that the potentiating effects of (±)Bay K 8644 develop gradually, as the depolarization time elapses, as if the dihydropyridines were acting through the same mechanism to accelerate or delay it. Remember that after alternating periods of depolarizing and resting conditions, recovery of secretion is greater and greater (Fig. 2); in this sense, (±)Bay K 8644 seems to mimic this recovery phenomenon.

DISCUSSION

Before establishing any firm conclusion, the crucial question which has to be answered is whether functional data presented here truly reflect changes in the kinetics of a given Ca^{2+} channel. It is unquestionable that direct measurements of whole-cell or single-channel Ca^{2+} currents using modern patch-clamp techniques (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) could give a direct answer to whether a given type of Ca^{2+} channel is present in cat chromaffin cells. But it is also true that using these techniques (which are not yet available in our laboratory) we would hardly correlate these data with secretion in intact glands. This is also true even for intact single cells. As far as we know, measurement of secretion in the millisecond range from a single chromaffin cell is not yet technically feasible; even the measurement of capacitance steps, reflecting the summed result of many exocytotic vesicular fusion events, is a very slow technique (various seconds) to correlate catecholamine release with current kinetics (Clapham & Neher, 1984).

Additional reasons to justify the present experimental approach in the cat adrenal gland is the absence of electrophysiological studies on its chromaffin cells, which exhibit an exquisite sensitivity to 1,4-dihydropyridine derivatives. In the widely used ox chromaffin cells, several patch-clamp studies on Ca^{2+} currents are available; however, clear-cut differences in functional responses between bovine and feline adrenal glands (see Introduction) preclude extrapolation of those findings to the cat. While Ca^{2+} uptake and secretion show a heterogeneous and complex pattern in the ox, the cat displays a more homogeneous and simple picture. For instance, K^+-evoked Ca^{2+} uptake and catecholamine release are clearly potentiated by Bay K 8644 and fully inhibited by various dihydropyridines (see Introduction). Therefore, correlation of these functional parameters with the activity of a putative homogeneous population of Ca^{2+} channels seems easier in the cat than in the ox chromaffin cell.

In spite of those considerations, we are aware that inferences on the kinetics of Ca^{2+} channels in cat chromaffin cells cannot be made without direct recordings of Ca^{2+} currents with patch-clamp techniques. However, it is plausible that in the intact adrenal medulla, inactivation of all Ca^{2+} channels in all chromaffin cells is not taking place simultaneously. By monitoring $^{45}Ca^{2+}$ uptake and secretion, we are measuring averaged events taking place in all cells; this information cannot be obtained with patch-clamp techniques, yet it is highly relevant to the understanding of the physiology of the adrenal gland working as an integrated organ. On the other hand, the higher rates of perfusion that we are using (4- to 8-fold higher than previous studies), the short pulses of Ca^{2+} or Ba^{2+} that we apply to trigger exocytosis (1–10 s) and the fact that the time dependence of the inactivation and reactivation of

secretion is in the 'few-seconds' range are factors not far away from the slow kinetics of L-type Ca^{2+} currents in general (Tsien, Lipscombe, Madison, Bley & Fox, 1988) and those of chromaffin cells in particular (Fenwick *et al.* 1982). Therefore, we feel that we are entitled to extrapolate, with the necessary caution, our present findings to the feline chromaffin cell Ca^{2+} channels. We hope to be able in the near future to corroborate them with patch-clamp techniques.

Evidence that restriction of calcium uptake through calcium channels is responsible for the inactivation by depolarization of catecholamine release

Various facts indicate that changes in the rates of secretion induced by depolarization are mediated by parallel changes in the rates of opening and closing of voltage-dependent Ca^{2+} channels.

(1) Cd^{2+} completely blocked secretion in the absence of depolarization. It is known that Cd^{2+} is a potent inorganic Ca^{2+} channel blocker in various excitable cells (Tsien *et al.* 1988).

(2) Ca^{2+} uptake into adrenal medullary chromaffin cells is blocked by depolarization to the same extent as catecholamine release.

(3) The Ca^{2+} channel activator (±)Bay K 8644 prevents the time-dependent inactivation, and the blocker (+)PN200-110 accelerates it.

We have to add to the present evidence data showing that nitrendipine (Artalejo *et al.* 1988*b*) and (+)PN200-110 (Borges, Ballesta & García, 1987; Fonteríz, Gandía, López, Artalejo & García, 1987) reduced drastically K^+-evoked $^{45}Ca^{2+}$ uptake into chromaffin cells from intact cat adrenal glands at nanomolar concentrations.

The subtype of calcium channel involved in secretion

Investigations of single-channel events using patch-clamp techniques have identified a variety of electrically operated channels controlling specifically the Ca^{2+} conductance of plasma membranes of several excitable cells. Though three main types (L, T and N) of Ca^{2+} channels have been characterized (Tsien *et al.* 1988), other subtypes have also been demonstrated recently (Kostyuk, 1989). From their activation threshold or their absolute conductances we cannot guess which of these channels might be responsible for regulating secretion in the cat chromaffin cell. However, the inactivation pattern or the sensitivity of Ca^{2+} current to drugs could tentatively indicate the subtype of Ca^{2+} channel involved. Rapidly inactivating low-threshold channels of the T type cannot be mediating secretion because of the following facts. (i) They inactivate very quickly at strong depolarizations; therefore, these channels will be quickly inactivated in conditions such as those used here with 118 mM-K^+. (ii) Their conductances are similar using Ca^{2+} or Ba^{2+}, but here the rates of secretion were substantially higher with Ba^{2+} than with Ca^{2+} (see Figs 5 and 6). (iii) They are insensitive to dihydropyridines. Also, N-type Ca^{2+} channels, which are known to be resistant to dihydropyridines, are unlikely to be involved in the regulation of secretion from cat chromaffin cells.

So, L-type, high-threshold, slowly inactivating Ca^{2+} channels, which are sensitive to dihydropyridines and Cd^{2+} and exhibit higher conductances to Ba^{2+} than to Ca^{2+} (Kostyuk, 1989), are likely to be responsible for the control of secretion in the feline chromaffin cell.

The L-type calcium channel controlling secretion in cat chromaffin cells inactivates in a voltage- but not calcium-dependent manner

On the basis of voltage-clamp experiments performed in *Paramecium* and *Aplysia* neurons, Brehm & Eckert (1978) and Tillotson (1979) suggested that during depolarization, Ca^{2+} accumulates in the cytosol as a result of Ca^{2+} entry and produces a specific inhibitory effect on Ca^{2+} channel conductance that appears as inactivation. This hypothesis claims that the cytosolic Ca^{2+} increase is a necessary as well as a sufficient cause of inactivation of Ca^{2+} channels and that voltage changes play, if anything, a minor role. Though initially these authors suggested that perhaps all Ca^{2+} channel inactivation is induced by Ca^{2+} entry into cells, Ca^{2+} currents that are primarily inactivated by changes in membrane potential (Fox, 1981) or by a mixture of voltage and Ca^{2+} have also been described (Hagiwara & Byerly, 1981; Tsien, 1983).

The interpretation of the inactivation of secretion in the cat chromaffin cell is hampered by two facts: (i) that in bovine chromaffin cells Ca^{2+} uptake (Artalejo *et al.* 1987) and 'standard' Ca^{2+} currents (Hirning *et al.* 1989) are inactivated in a Ca^{2+}- but not voltage-dependent manner; and (ii) that various subtypes of Ca^{2+} channels seem to be present at least in bovine chromaffin cells. However, if, as we believe, and in contrast to the ox chromaffin cell, a single, L-type Ca^{2+} channel is controlling any secretory response in cat adrenal chromaffin cells, its pure inactivation by voltage is consistent with all of our following findings:

(1) The higher the K^+ concentration (35 or 118 mM) present in the Ca^{2+}-free depolarizing Krebs solution, the faster the inactivation of secretion.

(2) Any of the experimental protocols used here avoids the previous accumulation of Ca^{2+} in the cytosol prior to the Ca^{2+} secretory test pulse. Glands are continuously perfused, for several hours, with Ca^{2+}-free solutions. In fact, Ca^{2+} introduced during the secretory tests was given for short times (1 and 10 s) precisely to avoid its accumulation in the cytosol; in addition, the fact that inactivation develops gradually depending upon the time of depolarization, yet the Ca^{2+} pulse always lasts for 10 s, precludes any association between this Ca^{2+} and inactivation.

(3) In spite of the fact that Ba^{2+} permeates Ca^{2+} channels better than Ca^{2+} in various excitable cells (Hagiwara & Byerly, 1981) including chromaffin cells (Artalejo *et al.* 1987), and that Ba^{2+} produces a higher rate of secretion initially, the rates of inactivation are similar with Ca^{2+} and Ba^{2+}. This is inconsistent with Ca^{2+}-dependent inactivation, where the kinetics of the inactivation process change greatly with Ba^{2+}; for instance, K^+-induced Ba^{2+} uptake does not inactivate in bovine chromaffin cells while Ca^{2+} uptake inactivates quickly (Artajelo *et al.* 1987).

Mechanisms of inactivation of L-type calcium channels controlling secretion in cat chromaffin cells

Because Ca^{2+} currents disappear in dialysed cells it is believed that, contrary to others, Ca^{2+} channels require a continuous metabolic support for their normal functioning; the end-point of this regulatory mechanism could be the phosphorylation of some components of the channel-forming proteins (Chad & Eckert, 1986). Because increases in cytosolic Ca^{2+} have been associated with dephosphorylation of some proteins through the activation of Ca^{2+}-dependent phos-

phatases, it has been suggested that dephosphorylation of the channel-forming protein might be responsible for Ca^{2+}-dependent inactivation of Ca^{2+} channels (Kameyama, Hescheler, Mieskes & Trautwein, 1986).

Being Ca^{2+}-independent, it seems logical to conclude that in the inactivation of L-type Ca^{2+} channels and secretion in feline chromaffin cells, these phosphorylating mechanisms are unlikely to mediate such phenomena. In contrast to the fast inactivation of the Na^+ channel, where voltage produces a quick change in its gating properties, L-type Ca^{2+} channels in the cat chromaffin cell inactivate much more slowly. Therefore, it seems clear that a Ca^{2+}-independent, but voltage-dependent biochemical mechanism is involved in the regulation of its gating.

Such a mechanism must be readily reversible, and probably requires intact cells. In fact, various cycles of inactivation and reactivation of secretion take place in the same gland throughout several hours of experiment. As we know that dihydropyridine derivatives either prolong or shorten the open time of L-type Ca^{2+} channels, and high-affinity binding sites for [^{3}H](+)PN200-110 (Castillo *et al.* 1989) and [^{3}H]nitrendipine (García *et al.* 1984; Ballesta *et al.* 1989) have been identified in bovine and cat (unpublished results, J. J. Ballesta and A. G. García) cell membranes, we are tempted to ascribe such a regulatory role to an endogenous dihydropyridine-like substance manufactured by chromaffin cells themselves. In fact, we already formulated this hypothesis when we first used Bay K 8644 in cat and ox chromaffin cells (García *et al.* 1984). This endogenous modulator could be a Bay K 8644-like substance whose production might be decreased in depolarized cells and increased in hyperpolarization; the reverse could be true for a dihydropyridine-like blocker. In fact, an endogenous factor which displaces the binding of [^{3}H]nitrendipine to membranes, activates heart Ca^{2+} currents and blocks neuronal currents, has been isolated recently from rat brain extracts (Callewaert, Hanbauer & Morad, 1989).

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