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Dopamine induces intracellular Ca^{2+} signals mediated by α_{1B}-adrenoceptors in rat pineal cells

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Abstract

We have studied the functional interaction of dopamine with α_1-adrenoceptor subtypes by measuring intracellular Ca^{2+} levels in pineal cells, a cell type where adrenoceptors are well characterized. We show that dopamine induces transient intracellular Ca^{2+} signals in only 70% of cells responding to phenylephrine. Dopamine-induced Ca^{2+} signals desensitise faster than Ca^{2+} transients elicited with phenylephrine and are selectively blocked by desipramine, imipramine, and α_{1B}-adrenoceptor antagonists. These results suggest that dopamine induced Ca^{2+} signals are mainly due to the activation of one subtype of α-adrenoceptor, the α_{1B}.

Keywords: Dopamine; α-Adrenoceptor; Pineal cell; Catecholamine

1. Introduction

Noradrenaline binds at least to 10 different adrenoceptors, which either activate or inhibit adenylate cyclase, or are coupled to phospholipase C. All adrenoceptors share three conserved residues within their transmembrane segments, which are thought to be the minimal structure for ligand binding. This residues are aspartate in the third transmembrane segment which may act as a counterion for the amine moiety in biogenic amines, and two serines in the fifth transmembrane segment thought to be involved in the recognition of the two hydroxyl groups of adrenaline and noradrenaline (Strader et al., 1987, 1989).On the other hand, dopamine binds to a different family of receptors formed by at least five subtypes. Dopamine receptors, which are mainly coupled to the activation or the inhibition of adenylate cyclase, share with adrenoceptors the same three conserved residues within their transmembrane segments for the ligand binding. This is not surprising given the structural similarity between adrenaline, noradrenaline and dopamine.

The possibility that all three catecholamines may indistinctly activate dopamine receptors and adrenoceptors has been long timed suspected. So far, β-adrenergic-like effects of dopamine have been demonstrated both on the vascular system and the heart (Rajfer et al., 1988; Ouedraogo et al., 1998). Similarly, in human platelets, it has been shown that dopamine increases intracellular cAMP levels through the activation of β-adrenoceptors (Anfossi et al., 1993) and, in rat adipocytes, dopamine lowers glucose uptake through the activation of β_3-adrenoceptors (Lee et al., 1998).

We have studied the effect of dopamine on intracellular Ca^{2+} levels in rat pinealocytes, a cell type having α- and β-adrenoceptors and where the intracellular pathways activated by noradrenaline have been deeply studied. Our results show that dopamine, at low micromolar concentrations, is able to elicit intracellular Ca^{2+} increments due to the activation of α-adrenoceptors. The pharmacological characterisation of receptors mediating the Ca^{2+} increment triggered by dopamine points to the preferential activation of the α_{1B}-adrenoceptor over other α_1 subtypes.

2. Materials and methods

2.1. Cell dissociation

Pineal glands were obtained from male Wistar rats (100–150 g) housed in a temperature-controlled room

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(23 ± 3 °C) and maintained on a 12:12-h light–dark cycle (60–80 μW cm^{-2}). For each experiment, six glands were washed and cleaned in cold Ringer solution (containing in mM: NaCl, 145; KCl, 2.7; and HEPES, 10; pH 7.25 NaOH), and transferred to fresh solution with 0.5 mg/ml protease where they were shaken for 30 minutes. The tissue was then washed twice in Dulbecco's Modified Eagle's Medium (BioWhittaker, Verviers, Belgium) supplemented with 5% v/v foetal bovine serum, 50 u.i./ml penicillin and 50 μg/ml streptomycin, mechanically dispersed in clean medium and plated onto poly-L-lysine coated cover-slips. Cells were kept in a CO_2 incubator (5%) at 36.5 °C until use (1–3 days later).

2.2. Solutions and chemicals

Ringers and test solutions were continuously applied by superfusion. The bars marked on the figures indicate the time at which the solutions were switched (dead time 2–5 s). The composition of the solution used was (in mM): NaCl, 145; KCl, 2.7; $CaCl_2$, 10; $MgCl_2$, 1 and HEPES, 10; glucose 10–20, pH 7.25–7.35 (NaOH). In some experiments, $CaCl_2$ was omitted and 1 mM EGTA added to the solution. Ascorbic acid (5 mM) was included in all solutions to prevent catecholamine oxidation. Salts were purchased from Sigma (Madrid, Spain), agonists and antagonists were obtained from Research Biochemicals International (Natik, MA, USA). Drugs were prepared immediately before use. All experiments were performed at room temperature (20–22 °C).

2.3. Ca^{2+} measurements

Measurements of intracellular Ca^{2+} in single cells were done using the acetoxymethyl (AM) ester, membrane permeable form, of the Ca^{2+} indicator dye Fura-2 (Fura2/AM). Cells were incubated during 5–10 min with 2 μM Fura 2/AM (Molecular Probes, OR, USA) at 36.5 °C and subsequently washed in Fura-2-free solution for a minimum of 10 min. Ca^{2+} measurements, carried out on the stage of an inverted microscope (Zeiss, Axiovert 35), were done as described by Almers and Neher (1985) and Marín et al. (1996). Briefly, the light from a 75-W Xenon arc lamp was alternately filtered by means of a rotating wheel at 357 and 380 nm. The filtered light was guided through a filter transmission 430-nm dichroic mirror and its emission passed through a long pass 470-nm filter before projection onto the photomultiplier tube. $[Ca^{2+}]_i$ was calculated on-

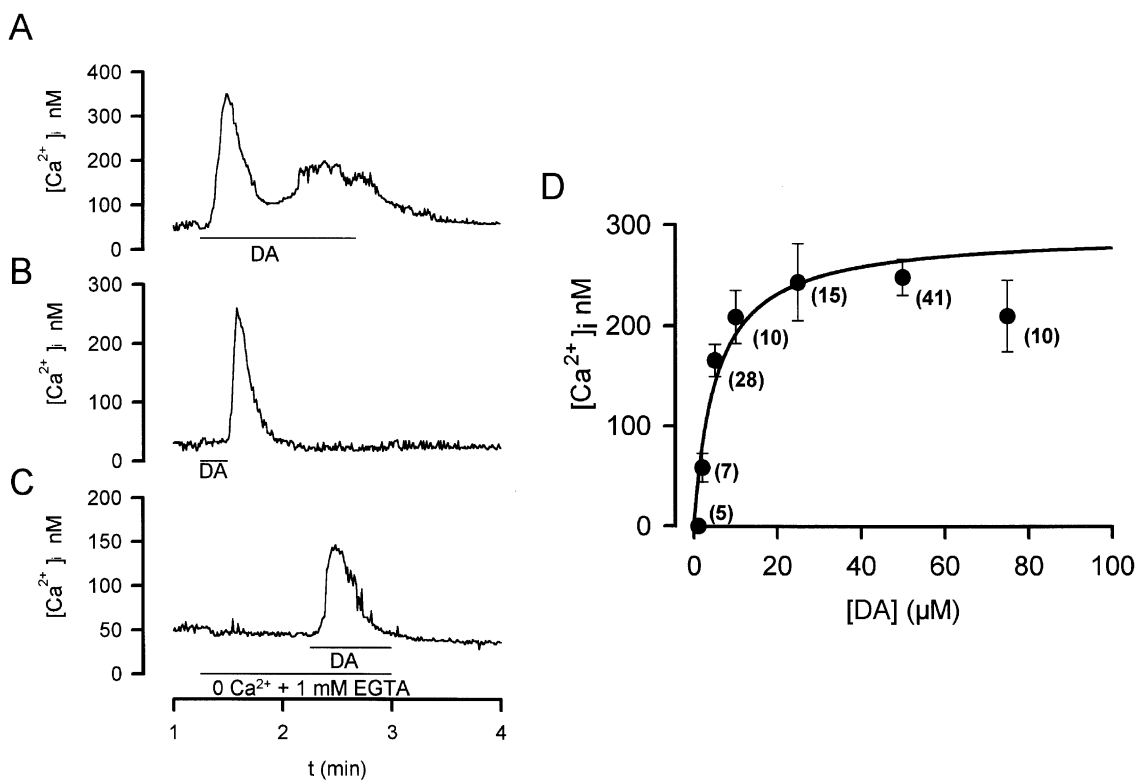

Fig. 1. Dopamine (DA) induced intracellular Ca^{2+} increments in pineal cells. (A) Prolonged dopamine exposure produced a response consisting of a transient peak that decline to a sustained phase above the basal level. (B) Short dopamine exposure elicited only the first peak. (C) In the presence of EGTA (1 mM), and without Ca^{2+}-added in the extracellular solution, the transient response was maintained, whereas the plateau was not elicited. (D) Relationship between the amplitude of the Ca^{2+} spike and dopamine concentration. The curve was fitted by Michaelis–Menten type equation of the form: $Ca^{2+} = Ca^{2+}_{max}[DA]/[DA] + K_d$, $n = 116$, $K_d = 5$ μM. dopamine concentration in A, B and C was 5 μM; recordings from three different cells.

line from the ratio of the emission at 357-nm excitation to that at 390-nm according to Grynkiewicz et al. (1985).

3. Results

3.1. Dopamine induces Ca^{2+} mobilisation

The effect of dopamine exposure in pineal cells was analysed by single-cell Ca^{2+} recording. Dopamine induced transient intracellular Ca^{2+} signals in 132 out of 189 cells (70%) responding to phenylephrine, an α_1-adrenoceptor agonist that mediates Ca^{2+} increments in pineal cells (Sugden et al., 1987; Marín et al., 1996). Dopamine induced intracellular Ca^{2+} increments consisting of a transient peak followed by a plateau (Fig. 1A). The plateau phase was absent when dopamine was applied for ≤ 20 s (Fig. 1B) or when the recording was made in an extracellular solution without Ca^{2+}-added plus 1 mM EGTA ($n = 9$) (Fig. 1C), suggesting that the dopamine-induced transient response was mostly due to mobilisation of intracellular Ca^{2+}, whereas the sustained phase was dependent on extracellular Ca^{2+}.

The peak amplitude of the transient Ca^{2+} signal increased with dopamine concentration, in the range of 1–50 μM. The relationship between the amplitude of the Ca^{2+} spike and dopamine concentration was obtained from experiments performed in 116 cells (Fig. 1D). The mean amplitude of the Ca^{2+} signal at saturating dopamine concentrations ranged between 200 and 240 nM, and half maximal amplitude was reached at a dopamine concentration of 5 μM.

3.2. Dopamine receptor agonists do not induce Ca^{2+} mobilisation

In order to study the receptor types implicated in the dopamine-mediated Ca^{2+} responses, the effects of different dopamine receptor agonists were tested. Neither R(+)-SKF-38393 (25–200 μM, $n = 12$; Fig. 2A), a dopamine D_1-like receptor agonist, nor Quinpirole (100 μM, $n = 3$, e.g. Fig. 2B), a D_2-like dopamine receptor agonist, was able to elicit any response on intracellular Ca^{2+}. The same result was observed with either bromocriptine (50 μM, $n = 5$) or apomorfine (50 μM, $n = 4$), dopamine D_2-like and D_1/D_2-like receptor agonists, respectively. The lack of effect of dopamine receptor agonists on intracellular Ca^{2+} levels indicates that the Ca^{2+} responses induced with dopamine in pineal cells are not mediated through dopamine receptors.

3.3. Block of the dopamine-mediated Ca^{2+} increments with prazosin and other catecholamine receptor antagonists

In order to test whether dopamine-induced Ca^{2+} responses were affected or not by prazosin, an α_1-adrenoceptor antagonist, cells were exposed repetitively to dopamine (5 μM) in the presence or the absence of the antagonist. Dopamine-mediated Ca^{2+} signal was not affected by 1 nM prazosin (Fig. 3A and C), while it was completely abolished by a concentration of 10 nM (Fig. 3B and C). Within this range, Ca^{2+} response peak amplitudes were inversely proportional to the prazosin concentration used (Fig. 3C, $n = 16$). The percent of inhibition of the Ca^{2+} responses as a function of the prazosin concentration was calculated after correcting for the desensitisation component resulting from repetitive dopamine stimulation (white bars in Fig. 3C). The corrected values are represented in Fig. 3D (triangles). From the curve fitting of the data points, half inhibition of the response was obtained at 3 nM prazosin, a value which is close to K_i reported for α_1-adrenoceptors (0.14–1.2 nM) in rat and human tissues (O'Malley et al., 1998).

The effect of dopamine receptor antagonists on dopamine-mediated Ca^{2+} increase was also studied. Haloperidol, classically a dopamine D_2/D_1 receptor antagonist, and spiperone, an antagonist for D_2-type dopamine receptors, inhibited Ca^{2+} responses at relatively low concentrations. Fig. 3D shows the percent of inhibition of the Ca^{2+} response as a function of the antagonist concentrations. Half inhibition values were 15 nM with haloperidol

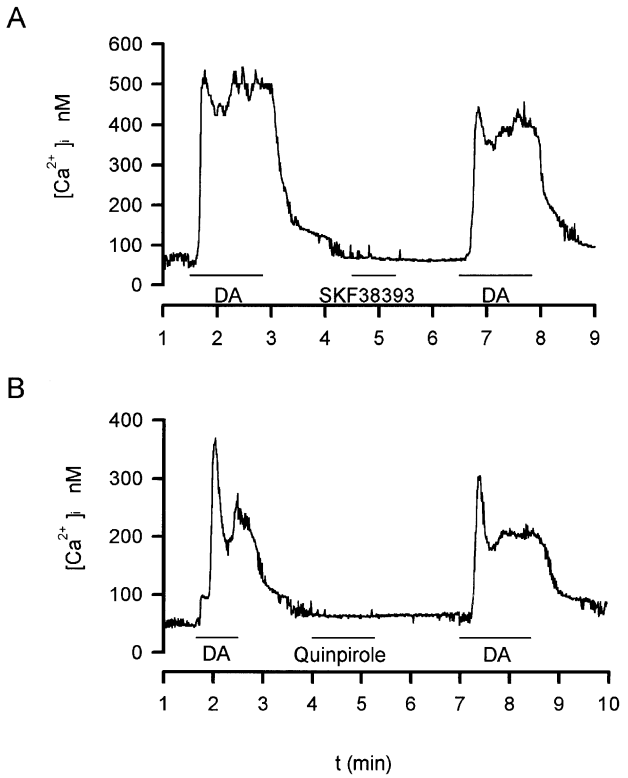

Fig. 2. Lack of effect on $[Ca^{2+}]_i$ of dopamine receptor agonists in two cells. Application of 50 μM SKF-38393 (A) or 100 μM quinpirole (B) did not induce Ca^{2+} responses, while exposure to 50 μM dopamine elicited robust signals; recordings from two different cells.

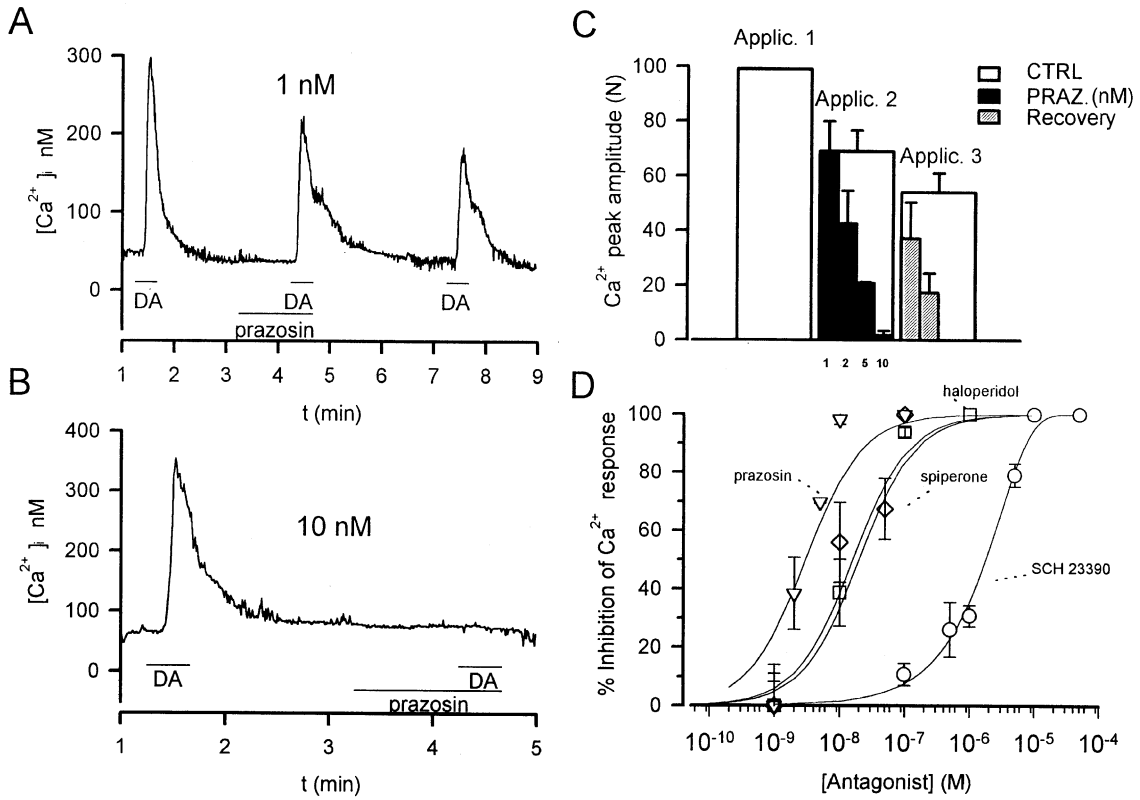

Fig. 3. Inhibition of dopamine-mediated Ca^{2+} signals by different catecholamine antagonists. (A, B) Responses to the application of dopamine (5 μM) before, during or after the application of prazosin at 1 nM (A) or 10 nM (B) in two cells. (C) Normalized peak Ca^{2+} amplitudes (mean ± S.E.M.) in response to three consecutive applications of dopamine (5 μM) in the presence or absence of prazosin. Sizes of white bars correspond to Ca^{2+} signal amplitudes in response to dopamine in control experiments without prazosin. Height of black bars represents the amplitudes of the Ca^{2+} responses to the second application of dopamine in the presence of different concentrations of prazosin (1, 2, 5 or 10 nM, $n = 16$). Strip bar amplitudes correspond to the recovery of the Ca^{2+} responses during the third application of dopamine after washing out prazosin. In all cases, dopamine exposures lasted 15–20 s and were repeated every 3 min. (D) Percent of inhibition of the Ca^{2+} signals elicited by dopamine (5 μM) as a function of the concentration of prazosin ($n = 16$), haloperidol ($n = 26$), spiperone ($n = 20$) and SCH-23390 ($n = 22$).

(Fig. 3D, squares, $n = 20$) and 20 nM with spiperone (Fig. 3D, diamonds). We also tested the effect of R(+)-SCH-23390, a D_1-type dopamine receptor antagonist, on dopamine-induced Ca^{2+} responses. This compound was much less efficient in producing inhibition of the response (Fig. 3D, circles), with an IC_{50} of 1.7 μM SCH-23390. Therefore, the higher inhibiting potency of prazosin, as compared to the others antagonists, suggests that the Ca^{2+} response to dopamine was mediated by the activation of α_1-adrenoceptors, and that both haloperidol and spiperone inhibited, with relatively high affinity, this receptor subtype. To test this last possibility, cells were stimulated with phenylephrine in the presence of different concentrations of haloperidol (Fig. 4). As with dopamine, haloperidol inhibited the Ca^{2+} responses to phenylephrine with a similar potency.

To study whether or not dopamine and phenylephrine activate the same subtypes of α_1-adrenoceptors, the effects of successive pulses of these agonists applied to the same cell were compared. In most cells, dopamine (5 μM) and phenylephrine (1 μM) elicited Ca^{2+} transients of similar form and duration, although the amplitudes of the Ca^{2+} signal obtained with dopamine were usually smaller that those obtained with phenylephrine (Fig. 5A). In 30% of the cells recorded, only phenylephrine elicited a Ca^{2+} response (Fig. 5B). No morphological differences were observed under the microscope between cells responding or not to dopamine. In cells responding both to dopamine and phenylephrine, experiments with repetitive applications of a high concentration of dopamine (50 μM) and a relatively low phenylephrine concentration (1 μM) revealed a different rate of desensitisation. Panels C and D in Fig. 5 show two examples where such a protocol was used. In all cases, the peak amplitude of the dopamine-mediated Ca^{2+} responses decreased with successive applications, while those induced by phenylephrine were maintained ($n = 5$). A negative effect of dispersion enzymes on membrane receptors was rejected since the same results were obtained in one experiment in cells that had not been treated with protease. These results suggest that dopamine and phenylephrine activate different α_1-adrenoceptor subtypes.

To test further this possibility, we explored the effect of different selective α_1-adrenoceptor antagonists on dopa-

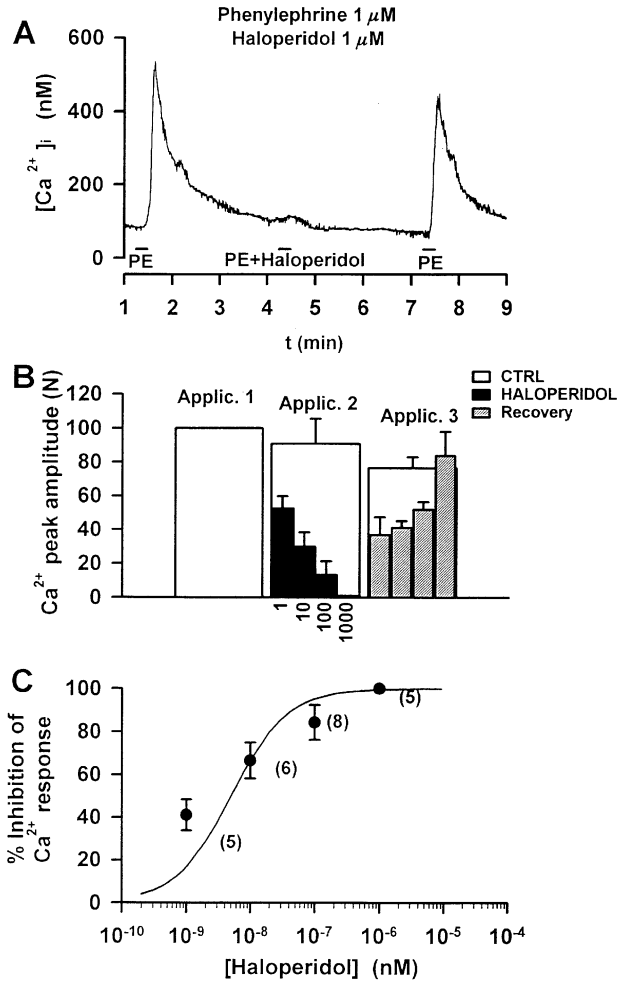

Fig. 4. Intracellular Ca^{2+} responses to phenylephrine (PE). (A) Responses to the application of phenylephrine (1 μM) before, during of after the application of haloperidol (1 μM) in one cell. (B) Normalized peak Ca^{2+} amplitudes (mean ± S.E.M.) in response to phenylephrine (15–20 s exposure, every 3 min.) in control (white bars), in the presence of haloperidol (1, 10, 100 or 1000 nM) during the second application of phenylephrine (black bars, $n = 24$), and in respond to the third application of phenylephrine after washing out haloperidol (stripped bars, n = 24). (C) Percent of inhibition of the Ca^{2+} signals as a function of the concentration of haloperidol ($n = 24$).

mine-mediated Ca^{2+} responses. Risperidone is about 120-fold more selective for α_{1B}-adrenoceptors ($K_i = 2.3$ nM) than for α_{1A}-adrenoceptors ($K_i = 280$ nM) (Sleight et al., 1993). In our experiments with risperidone, the IC_{50} was < 10 nM when the response was elicited by dopamine, and about 25 times higher when the stimulus was phenylephrine. On the contrary, WB4101 and benoxathian have been reported to have much higher affinity for α_{1A}-than for α_{1B}-adrenoceptors (Sugden et al., 1996; Yan et al., 1998). In our experiments, when the stimulus was dopamine, the IC_{50} with WB4101 was 10 nM, and 1 nM with benoxathian, in the range of that expected for α_{1B}-adrenoceptors (Sugden et al., 1996).

To explore further if the dopamine-induced Ca^{2+} increments were mediated by α_{1B}-adrenoceptors, cells were incubated with either 10 or 50 μM chloroethylclonidine, an alkylating agent, preferentially of α_{1B}-adrenoceptors, during 10–60 min. Thereafter, the cells were washed and stimulated either with dopamine (5 μM) or phenylephrine (1 μM). In those cells stimulated with dopamine, the inhibition of the response was complete with 50 μM chloroethylclonidine (Fig. 6A). A minor concentration of chloroethylclonidine, 10 μM, decreased the response to 44%. In cells stimulated with phenylephrine, the inhibition with 10 and 50 μM chloroethylclonidine was less, 49.7% and 69.3%, respectively (Fig. 6B). These results also suggest that dopamine is more selective activating α_{1B}-adrenoceptors than phenylephrine, which has been reported to be similarly potent at all three α_1-adrenoceptors subtypes (Minneman et al., 1994).

Besides α_{1B}-adrenoceptor antagonists, desipramine also inhibited the dopamine-mediated Ca^{2+} response ($n = 26$, Fig. 7). This drug is used in clinic as antidepressant, possibly due to its inhibitory effect at low concentrations on serotonine and noradrenaline transporters (Buck and Amara, 1994). In our experiments, the inhibition of the dopamine effect on intracellular Ca^{2+} by desipramine was concentration-dependent in the range 100–1000 nM ($IC_{50} = 250$ nM), while no inhibition was obtained within this range when the stimulus was phenylephrine or noradrenaline (Fig. 7C–E). Imipramine, other tricyclic antidepressant, at 1000 nM inhibited 90% of the response to dopamine, while it did not affect the response to phenylephrine ($n = 15$, data not shown).

4. Discussion

In this work, we describe the capability of dopamine to trigger Ca^{2+} signals in pineal cells. Ca^{2+} transients resulted from the release of Ca^{2+} from intracellular stores and the subsequent Ca^{2+} entry to the cytosol from the extracellular medium (Fig. 1). Half maximum responses were obtained with 5 μM dopamine. This value is within the activation range of dopamine receptor subtypes D_1 and D_2, but higher than the EC_{50} for receptor subtypes D_4 and D_5 (submicromolar affinity), and much higher than the EC_{50} for subtype D_3 (nanomolar affinity) (Gingrich and Caron, 1993). Although all cloned dopamine receptors are known to be only coupled to adenylate cyclase, there are several reports describing the activation of the phosphatidylinositol turnover and the generation of Ca^{2+} signals by dopamine in some tissues (Kebabian and Calne, 1979; Stoof and Kebabian, 1981; Onali et al., 1984; Felder et al., 1989a,b; Mahan et al., 1990; Vallar et al., 1990; Undie and Friedman, 1990). In bovine pineal cells, D_1 and D_2 dopamine receptors have been identified with radioligand binding assays (Govitrapong et al., 1984; Simonneaux et al., 1990). However, our results point against the presence of dopamine receptors coupled to phospholipase C in rat pinealocytes since, in our hands, the dopamine

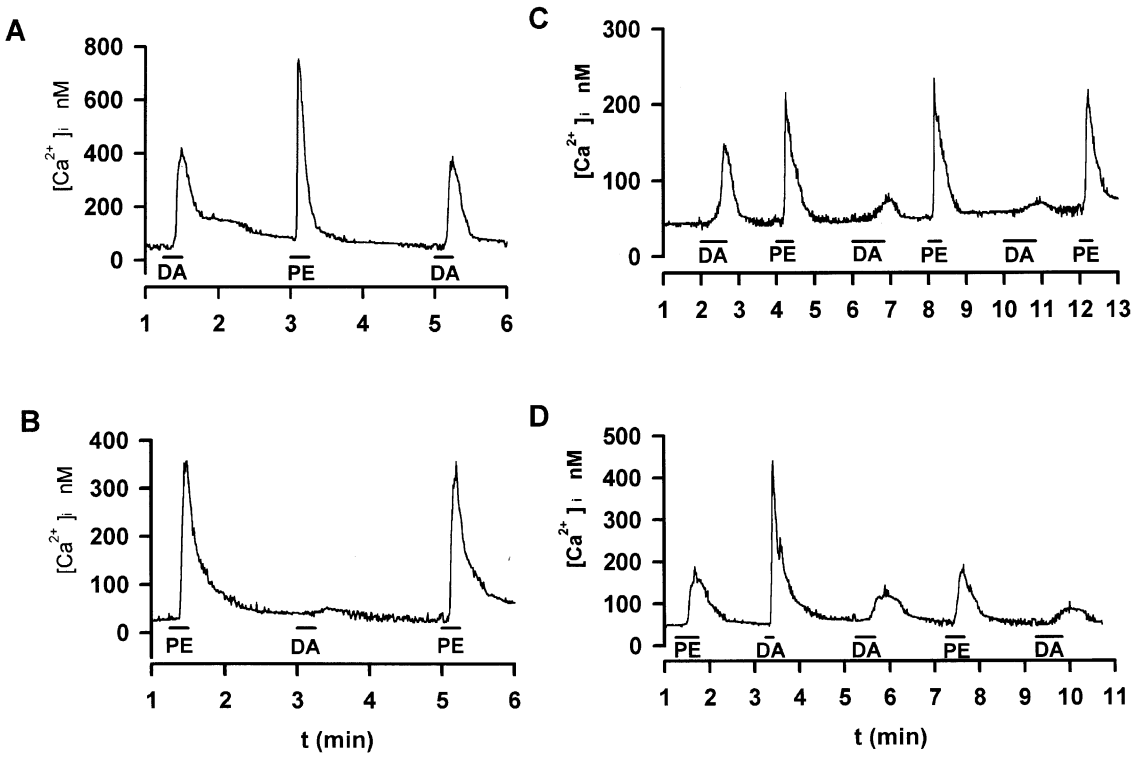

Fig. 5. Intracellular Ca^{2+} responses to dopamine and phenylephrine. (A) Example of a cell that responded to dopamine (5 μM) and phenylephrine (1 μM). (B) Example of a cell that only responded to phenylephrine. (C, D) Recordings from two cells with fast desensitisation to dopamine (50 μM) and not to phenylephrine (1 μM).

receptor agonists SKF-38393, bromocriptine, quinpirole and apomorphine, at saturating concentrations, were inefficient to increase intracellular Ca^{2+}. Furthermore, the selective α_1-adrenoceptor antagonist prazosin inhibited with high affinity (low nanomolar) the Ca^{2+} increment mediated by dopamine. The fact that some dopamine receptor antagonists (haloperidol, spiperone, or SCH-23390) also inhibited the effect of dopamine, although at a lower affinity than that of prazosin, is not surprising given the capability of these drugs to also bind to α_1-adrenoceptors. It must be pointed out that the opposite has also been described (i.e. the activation of dopamine receptors by noradrenaline) since in cat retinal ganglion cells, noradrenaline has been shown to activate D_2 dopamine receptors (Robbins et al., 1988).

The finding that in all cells tested the α_1-adrenoceptor agonist phenylephrine induced Ca^{2+} release from intracellular stores, but not all cells increased intracellular Ca^{2+} in response to dopamine, suggests that dopamine action is mainly mediated through the activation of an α_1-adrenoceptor subtype. This hypothesis was further confirmed by desensitising experiments. With successive applications of saturating dopamine concentrations, the Ca^{2+} responses progressively decreased, while those elicited with phenylephrine were maintained or decreased with a much slower time course. To ascertain what α_1-adrenoceptor subtype was mainly activated by dopamine, we used both the alkylating agent chloroethylclonidine, which preferentially inactivates α_{1B}-adrenoceptors, and three specific subtype antagonists. The results obtained suggest that dopamine mainly activates α_{1B}-adrenoceptors over other subtypes. In rat pineal cells, α_{1A}- and α_{1B}-adrenoceptors have been identified by radioligand binding and reverse transcription-polymerase chain reaction analysis (Sugden et al., 1996), which is compatible with our results. However, whether normally both receptor subtypes do exist in all pinealocytes or whether in some cells only one subtype is expressed, thereby resulting in a heterogeneous population of pinealocytes, remains to be further clarified.

A physiological role of dopamine on rat pineal cells has previously been proposed. In bovine pineal glands, dopamine is thought to have a regulatory role on melatonin synthesis and secretion (Govitrapong et al., 1989; Simonneaux et al., 1991). The existence of pineal dopaminergic innervation from central origin has also been postulated based on the presence of tyroxine hydroxylase activity in rat pineal glands after bilateral denervation from the superior cervical ganglia (Hernandez et al., 1994). In addition, it has been suggested that not all the dopamine accumulated in sympathetic nerve endings innervating the pineal gland is further metabolised to noradrenaline. This suggestion is based on the finding that only part of the fibres

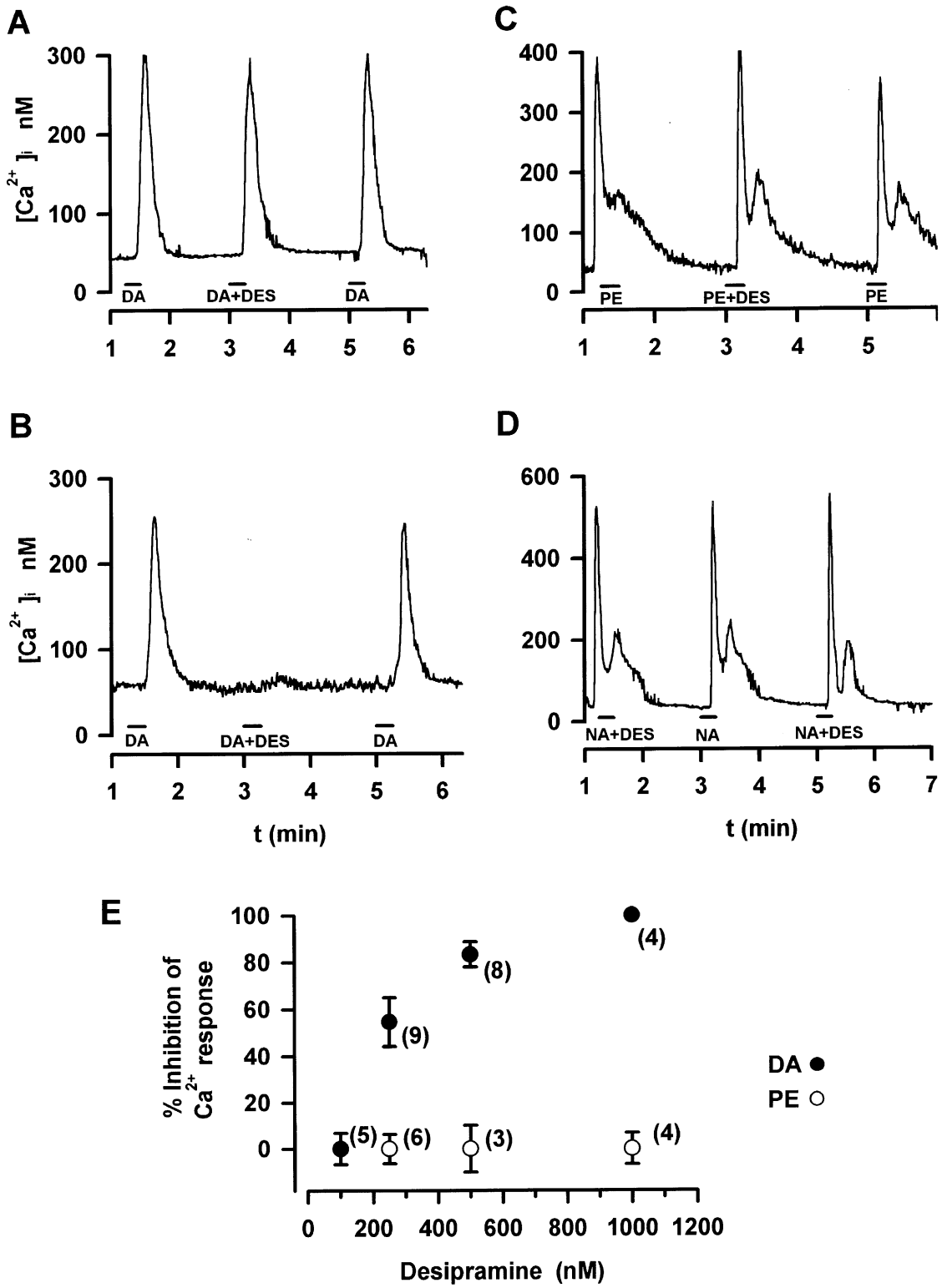

Fig. 7. (A, B) Intracellular Ca^{2+} responses to dopamine (5 μM) in presence or absence of desipramine (DES), 100 or 500 nM, respectively, in two cells. (C) Response to phenylephrine (1 μM) in the absence or presence of desipramine (500 nM). (D) Response to noradrenaline (NA) (1 μM) in the presence or absence of desipramine (1 μM). (E) Relationship between the inhibition of the response to catecholamines (dopamine 5 μM, IC_{50} = 250 nM, n = 26; phenylephrine 1 μM, IC_{50} > 1000 nM, n = 13) and desipramine concentration. In all cases, drug application lasted 15 s. Numbers in brackets represent number of experiments.

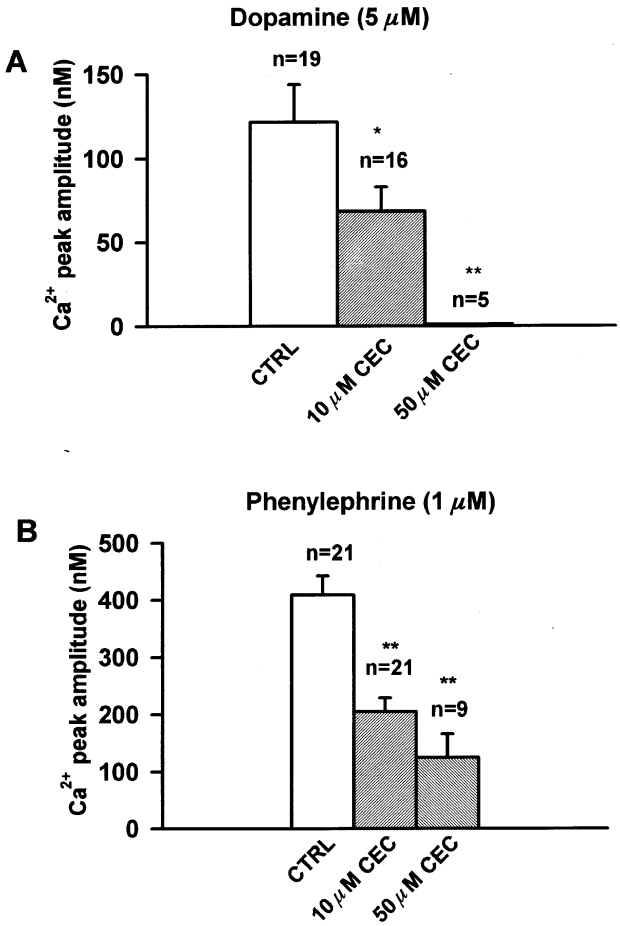

Fig. 6. Inhibition of catecholamines-mediated Ca^{2+} responses by 10 or 50 μM chloroethylclonidine. (A) Responses to the application of dopamine (5 μM) of cells preincubated with chloroethylclonidine ($n = 21$). (B) Responses to the application of phenylephrine (1 μM) of cells preincubated with chloroethylclonidine ($n = 30$).

displaying catecholamine fluorescence also shows dopamine-β-hydroxylase-like immunoreactivity (Schröder and Vollrath, 1985).

The fact that dopamine can activate adrenoceptors and that noradrenaline may also activate dopamine receptors *in vitro* opens the interrogation whether this phenomenon also occurs *in vivo*. In such case, the question emerges whether the term *cathecholaminergic receptors* has a genuine physiological meaning. On the other hand, specific functional interactions between different neurotransmitter receptor subfamilies have been recently reported (Liu et al., 2000). Taken together, these findings indicate that neurotransmitter-membrane receptor cross-talk may represent a more widespread phenomenon than previously expected.

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