Modulation of basal intracellular calcium by inverse agonists and phorbol myristate acetate in rat-1 fibroblasts stably expressing α_{1d}-adrenoceptors

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Abstract In rat-1 fibroblasts stably expressing α_{1d}-adrenoceptors BMY 7378, phentolamine, chloroethylclonidine and 5-methyl urapidil decreased basal $[Ca^{2+}]_i$. WB 4101 induced a very small effect on this parameter but when added before the other antagonists it blocked their effect. All these agents inhibited the action of norepinephrine. Phorbol myristate acetate also blocked the effect of norepinephrine and decreased basal $[Ca^{2+}]_i$. Staurosporine inhibited these effects of the phorbol ester. Our results suggest that: (1) α_{1d}-adrenoceptors exhibit spontaneous ligand-independent activity, (2) BMY 7378, phentolamine, chloroethylclonidine and 5-methyl urapidil act as inverse agonists and (3) protein kinase C activation blocks spontaneous and agonist-stimulated α_{1d}-adrenoceptor activity.

Key words: α_1-Adrenoceptor; Inverse agonist; Protein kinase C; Calcium

1. Introduction

α_1-Adrenoceptors are members of the G-protein-coupled family of receptors, which mediate some of the physiological actions of the natural catecholamines, epinephrine and norepinephrine. Three α_1-adrenoceptor subtypes have been cloned and they are referred to as α_{1a}-, α_{1b}- and α_{1d}-adrenoceptors, as recommended by the International Union of Pharmacology Committee for Receptor Nomenclature and Drug Classification [1]. These receptors are coupled to phosphoinositide turnover/calcium mobilization and, depending on the cell type, to other signaling devices [2,3].

It has been generally assumed that receptors are activated by agonists and that antagonists bind to the receptors without altering their activity but rather blocking the action of agonists. However, recent studies indicate that some receptors have spontaneous basal activity in the absence of any ligand, i.e. they can modulate downstream effector elements and induce functional responses without agonists [4–6]. In fact, it has been observed that some agents not only block the action of agonists but also suppress agonist-independent receptor activity; these agents are generally referred to as inverse agonists [4–6].

During our studies with rat-1 fibroblasts expressing the α_1-adrenoceptor subtypes, we observed that chloroethylclonidine behaved as a partial agonist for the α_{1a} subtype and antagonized the effect of norepinephrine in fibroblasts expressing either of the other two subtypes [7]. Interestingly, we consistently observed that chloroethylclonidine decreases the basal concentration of intracellular calcium ($[Ca^{2+}]_i$) in fibroblasts expressing the α_{1d} subtype [7]. We were puzzled by this finding and further investigated it. Our data show that other adrenergic antagonists induce this effect, suggesting that it is due to inverse agonism on agonist-independent activity of α_{1d}-adrenoceptors; in addition, it was observed that such endogenous activity can also be blocked by activation of protein kinase C.

2. Materials and methods

(−)Norepinephrine, endothelin-1, phorbol 12-myristate 13-acetate (PMA), staurosporine and bovine serum albumin were obtained from Sigma Chemical Co. Fura 2/AM was from Molecular Probes. Chloroethylclonidine, WB 4101, BMY 7378 and 5-methyl urapidil were from Research Biochemicals International. Phentolamine was a generous gift from Ciba-Geigy. Dulbecco's modfied Eagle's medium (DMEM), fetal bovine serum, trypsin, antibiotics and other reagents used for cell culture were from Gibco BRL.

Rat-1 fibroblasts stably expressing α_{1d}-adrenoceptors (cloned from rat brain [8]) were kindly provided to us by Drs. L. Allen, R.J. Lefkowitz and M.G. Caron (Duke University, Durham, NC, USA). Transfected cells were grown as described [9] in glutamine-containing high glucose DMEM supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, 100 U/ml penicillin and 0.25 μg/ml amphotericin B, at 37°C under a 5% CO_2 atmosphere. For selection, cells were cultured in the presence of the neomycin analogue, G418 sulfate (300 μg/ml).

Confluent cells were incubated overnight in G418-free DMEM without serum. Cells were loaded with 5 μM Fura 2/AM at 37°C for 1 h, then washed with Krebs-Ringer-HEPES buffer containing 0.05% bovine serum albumin, pH 7.4, detached from the plates with trypsin and washed three times, to remove unincorporated dye. Cells were resuspended in the same buffer, at a concentration of approximately 10^6 cells/ml. Fluorescence measurements were performed with excitation monochromators set at 340 and 380 nm, with a chopper interval of 0.5 s, and the emission monochromator set at 510 nm. The $[Ca^{2+}]_i$ was calculated according to Grynkiewicz et al. [10] using the software provided by AMINCO-Bowman; traces were directly exported to the graphs.

3. Results

Representative traces of the effects of norepinephrine and the selective α_{1d}-adrenergic antagonist BMY 7378 [11] on $[Ca^{2+}]_i$ are presented in Fig. 1. It can be observed that norepinephrine induced an almost immediate increase of $[Ca^{2+}]_i$; the increase was 2–3-fold the basal level in agreement with previous data [9]. As expected, BMY 7378 inhibited this action of norepinephrine in a concentration-dependent fashion, as evidenced by a gradual decrease in the magnitude of the response and the velocity of the increase (Fig. 1). Interestingly, BMY 7378 by itself decreased, also in a concentration-dependent fashion, the basal level of $[Ca^{2+}]_i$. The decrease was relatively small (30–50 nM) but it was consistent

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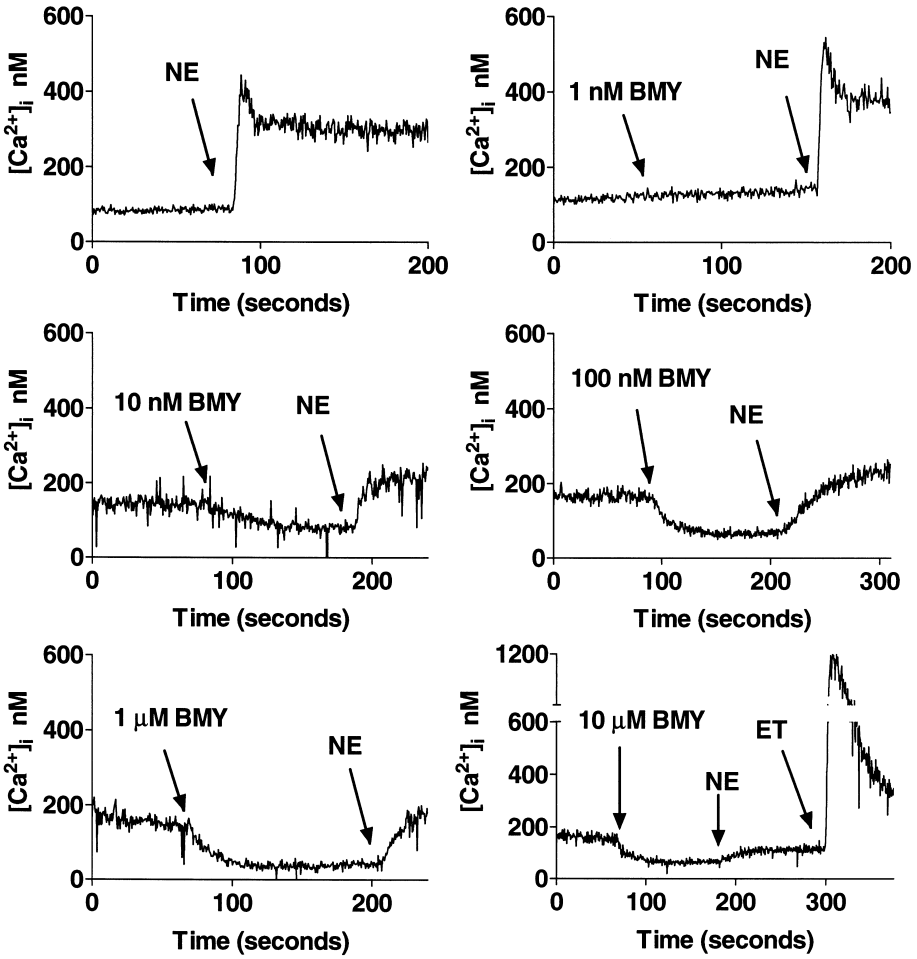

Fig. 1. Effect of adrenergic agents on intracellular calcium concentration ($[Ca^{2+}]_i$). Rat-1 fibroblasts expressing α_{1d}-adrenoceptors were incubated with 10 μM norepinephrine (NE) and the indicated concentrations of BMY 7378 (BMY). In the last trace the effect of 10 nM endothelin-1 (ET) is shown. Traces are representative of 4–6 experiments using different cell preparations.

and clearly evidenced by the gradual response in magnitude and speed (slope) (Fig. 1). In cells treated with 10 μM BMY 7378 there was a clear decrease in basal $[Ca^{2+}]_i$ and the effect of norepinephrine was almost abolished; however, the peptide mediator endothelin-1 induced a full response indicating that the action of BMY 7378 was not due to a general deleterious action on the cells but rather to a selective effect on the α_{1d}-adrenoceptors.

It can be observed that the antagonists WB 4101, 5-methyl urapidil, phentolamine, BMY 7378 and chloroethylclonidine inhibited in a concentration-dependent fashion the effect of norepinephrine on $[Ca^{2+}]_i$ (Fig. 2, upper left panel). Chloroethylclonidine only partially inhibited the effect of the catecholamine, even at the highest concentration tested (100 μM).

The effect of the different antagonists on basal $[Ca^{2+}]_i$ was studied. WB 4101 modified basal $[Ca^{2+}]_i$ to a very small extent (0–10 nM), whereas the other agents tested induced concentration-dependent decreases of basal $[Ca^{2+}]_i$ of 30–50 nM; 5-methyl urapidil consistently was less effective than phentolamine, BMY 7378 or chloroethylclonidine (Fig. 2).

We took advantage of the very small 'inverse efficacy' of WB 4101 and tested whether it could block the action of the other agents. As shown in Fig. 3, the previous addition of WB 4101 clearly blocked the effect of 5-methyl urapidil, phentolamine, BMY 7378 and chloroethylclonidine on basal $[Ca^{2+}]_i$.

Activation of protein kinase C with phorbol esters blocked α_1-adrenergic action [12,13], an effect associated with adrenoceptor phosphorylation [14–18]. Marked differences in sensitivity to the inhibitory action of protein kinase C activation have been observed among the receptor subtypes, α_{1d}-adrenoceptors being particularly sensitive [9]. We tested whether PMA was able to alter $[Ca^{2+}]_i$. As can be observed in Fig. 4, PMA decreased basal $[Ca^{2+}]_i$ in a concentration-dependent fashion; the decrease was of similar magnitude as that induced by the adrenergic antagonists tested. As expected, when the cells were treated with PMA the effect of norepinephrine was blocked but not that of endothelin-1 (Fig. 4). Staurosporine, an inhibitor of protein kinase C, blocked the effects of PMA on both basal $[Ca^{2+}]_i$ and the action of norepinephrine (Fig. 4). Similar action was observed with other inhibitors (data not shown).

4. Discussion

Our present data indicate that α_{1d}-adrenoceptors expressed in rat-1 fibroblasts exhibit spontaneous ligand-independent activity. Some agents such as 5-methyl urapidil, phentolamine,

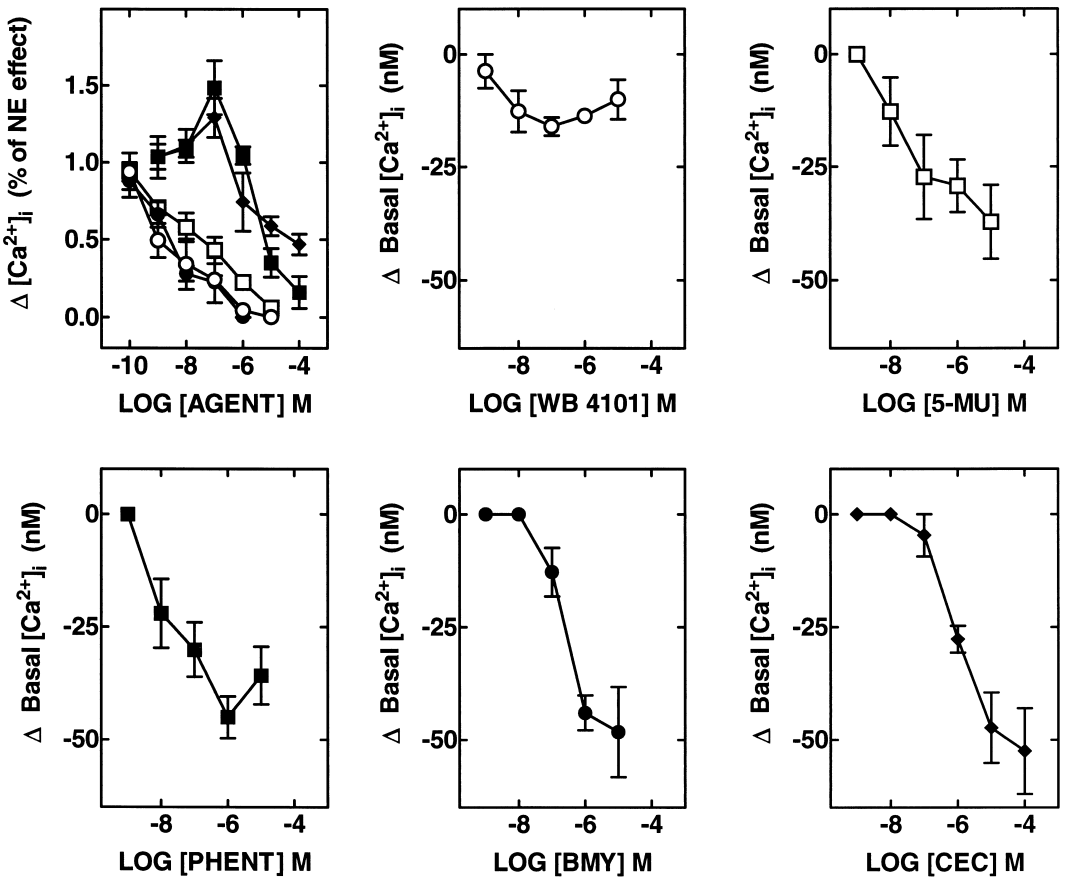

Fig. 2. Effect of adrenergic antagonists on basal and norepinephrine-stimulated intracellular calcium concentration ($[Ca^{2+}]_i$). Upper left panel: Cells were incubated with the indicated concentrations of the different antagonists, 2 min later 10 μM norepinephrine was added. Data were normalized to the effect of 10 μM norepinephrine alone (1=100% of norepinephrine effect). Other panels: The changes in the basal $[Ca^{2+}]_i$ observed during the addition of the antagonists are presented. Basal $[Ca^{2+}]_i$ was 155 ± 15 nM. The antagonists used were: WB 4101 (open circles), 5-methyl urapidil (open squares, 5-MU), phentolamine (solid squares, PHENT), BMY 7378 (solid circles, BMY) and chloroethylclonidine (solid diamonds, CEC). Plotted are the means and vertical lines represent the S.E.M. of 4–7 experiments using different cell preparations; when no S.E.M. are shown they are within the symbol.

BMY 7378 or chloroethylclonidine can block such activity, i.e. they seem to be acting as inverse agonists. This notion is supported by the fact that WB 4101, which is essentially devoid of such activity, can block the action of the inverse agonists as well as the effect of the agonist norepinephrine.

We observed some differences in the potency order for inhibiting the norepinephrine response as compared to that for decreasing basal $[Ca^{2+}]_i$. Such differences may be due to the fact that inverse agonism reflects not only drug affinity but also the ability of the ligand to stabilize the receptor in an inactive conformation. Differences may exist in inverse intrinsic activities that could modify the dose-response relationship, not only in efficacy but also in apparent potency, if receptor reserve exits.

The data obtained with PMA are consistent with this. Activation of protein kinase C by this phorbol ester blocks the increase in $[Ca^{2+}]_i$ induced by norepinephrine as well as the spontaneous agonist-independent receptor activity. The data suggest that the protein kinase C-mediated phosphorylation 'freezes' the α_{1d}-adrenoceptor in an inactive conformation. The fact that staurosporine blocks the decrease in $[Ca^{2+}]_i$ induced by PMA and also the ability of the active phorbol ester to inhibit the action of norepinephrine suggests that both events are likely mediated through the same process, i.e. receptor phosphorylation, mediated through protein kinase C activation.

The mechanism through which the spontaneously active receptor modulates $[Ca^{2+}]_i$ is not known. It may involve IP_3-mediated liberation of calcium from intracellular pools and/or modulation of calcium channels and extrusion mechanisms. This issue is far from trivial, especially considering that these fibroblasts express a calcium sensor that is a G-protein-coupled receptor [19]. In addition, it is likely that some adaptation mechanism(s) may be operating in these fibroblasts since the basal $[Ca^{2+}]_i$ is no different from that observed in non-transfected cells or in cells transfected with the other subtypes (data not shown).

It was previously observed that sustained treatment with α_1-adrenergic antagonists results in an up-regulation of α_{1b}-adrenoceptors in rat-1 fibroblasts expressing a constitutively active mutant but not the wild type receptor [20]. Interestingly, in this study phentolamine and WB 4101 had similar inverse activity [20]. Similarly it is interesting that chloroethylclonidine behaves as an antagonist in cells expressing α_{1b}-adrenoceptors, as a partial agonist in cells expressing the α_{1a} subtype [7] and as an inverse agonist in fibroblasts expressing α_{1d}-adrenoceptors.

To the best of our knowledge this is the first demonstration

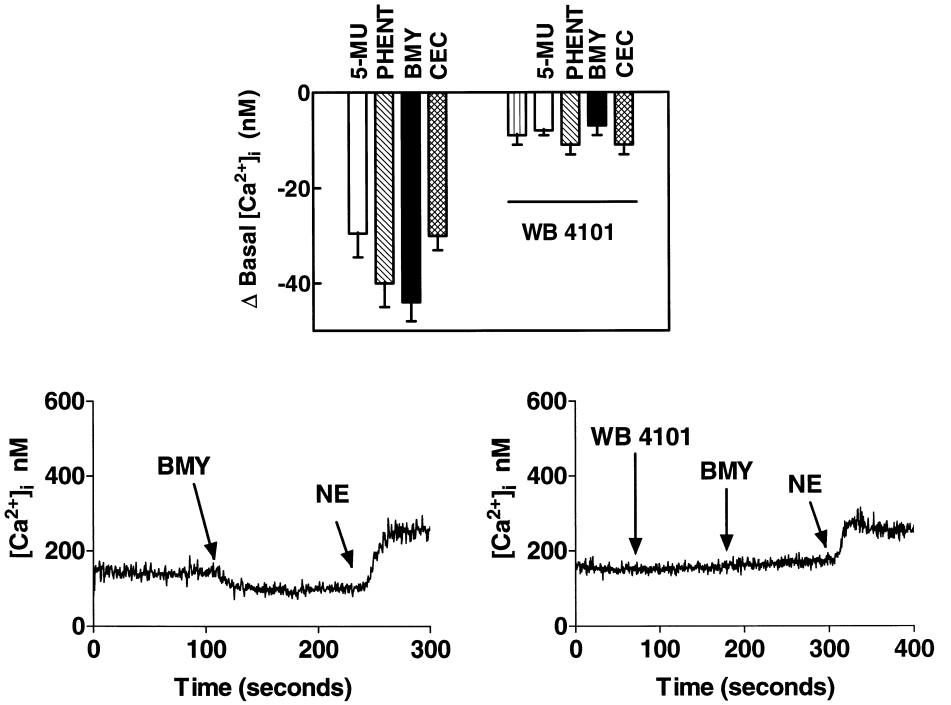

Fig. 3. Effect of WB 4101 on the effect of adrenergic antagonists on basal intracellular calcium concentration ($[Ca^{2+}]_i$). Upper panel: The changes due to the addition of 1 μM of the indicated agents are shown; where indicated WB 4101 was added alone or ~2 min before other agents and the total change in basal $[Ca^{2+}]_i$, i.e. that due to WB 4101 plus the other agent, is indicated. 5-Methyl urapidil (5-MU), phentolamine (PHENT), BMY 7378 (BMY), chloroethylclonidine (CEC). Plotted are the means and vertical lines represent the S.E.M. of 3–4 experiments using different cell preparations. Lower panels: Representative traces of the effect of antagonists on calcium concentration; the effect of 10 μM norepinephrine (NE) is also shown.

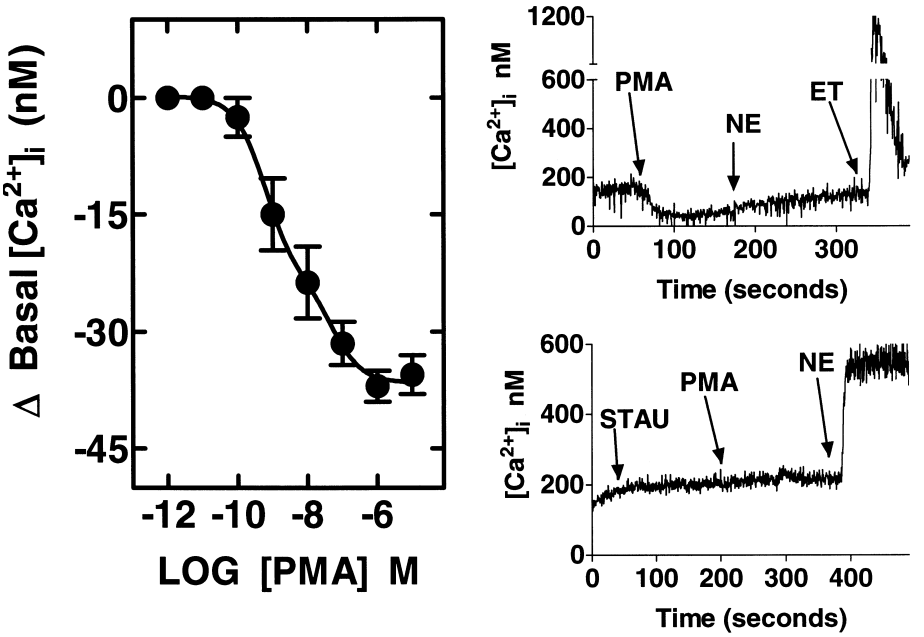

Fig. 4. Effect of PMA on basal intracellular calcium concentration ($[Ca^{2+}]_i$). Left panel: Effect of different concentrations of PMA on basal $[Ca^{2+}]_i$; plotted are the means and vertical lines represent the S.E.M. of 5–6 experiments using different cell preparations. Right panels: Representative traces of the effects of PMA and 1 μM staurosporine (STAU) on intracellular calcium concentration; the effects of 10 μM norepinephrine (NE) and 10 nM endothelin-1 (ET) are also shown.

of inverse agonism in this adrenergic subtype. Considering the important physiological (smooth muscle contraction) and physiopathological roles that this α_{1d}-adrenergic subtype seems to play in hypertension [21–24], the possibility that inverse agonists could be therapeutically useful seems worth considering.

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