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Effects of Phospholipase C Inhibitors on Ca^{2+} Channel Stimulation and Ca^{2+} Release from Intracellular Stores Evoked by α_{1A}- and α_{2A}-Adrenoceptors in Rat Portal Vein Myocytes

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The ability of phospholipase C inhibitors to inhibit Ca^{2+} channel stimulation and Ca^{2+} release from intracellular stores evoked by norepinephrine in single rat portal vein myocytes was investigated in the aim of identifying the type of phospholipase C involved in the transduction pathways activated by α_{1A}- and α_{2A}-adrenoceptors. U73122 (an inhibitor of phosphatidylinositol-phospholipase C) inhibited in a concentration-dependent manner the release of Ca^{2+} from the intracellular stores induced by activation of α_{1A}-adrenoceptors and related to inositol phosphate production whereas U73343 was ineffective. Both compounds had no effect on the release of Ca^{2+} induced by caffeine. However, U73122 and U73343 inhibited the L-type Ca^{2+} channel. D609 (an inhibitor of phosphatidylcholine-phospholipase C) had no direct inhibitory effects on the L-type Ca^{2+} channel but it inhibited concentration dependently the α_{2A}-adrenoceptor-induced stimulation of Ca^{2+} channels, which had been shown to be independent of phosphatidylinositol hydrolysis. Therefore, these results suggest that α_{2A}-adrenoceptors activate a phosphatidylcholine-phospholipase C in vascular myocytes. However, D609 had other sites of action as it blocked norepinephrine- and caffeine-induced Ca^{2+} release from the intracellular stores.

In vascular smooth muscle cells, activation of α_{1A}-adrenoceptors stimulates phospholipase C which hydrolyzes phosphatidylinositol-4,5-biphosphate to yield diacylglycerol (DAG) and inositol 1,4,5-trisphosphate ($InsP_3$). The receptors are coupled to phospholipase C through a trimeric GTP-binding protein (G protein) which has been identified to be G_q/G_{11} [1]. $InsP_3$ subsequently releases Ca^{2+} from the intracellular stores whereas DAG in concert with cellular Ca^{2+} activates protein kinase C (PKC). PKC may play a central role in phosphorylation of cellular proteins, including voltage-dependent Ca^{2+} channels [2, 3]. In contrast, activation of α_{2A}-adrenoceptors evokes stimulation of Ca^{2+} channels through a transduction pathway involving a $G_{i1\text{-}2}$ protein to activate PKC without $InsP_3$ production and release of Ca^{2+} from intracellular stores [4, 5]. The nature and identity of intermediates in these transduction pathways remain to be elucidated. In this study, therefore, we have used U73122 and D609 (inhibitors of phosphatidylinositol-phospholipase C and phosphatidylcholine-phospholipase C, respectively), to investigate their actions on the transduction pathways activated by norepinephrine.

MATERIALS AND METHODS

Wistar rats (150–160 g) were stunned and then killed by cervical dislocation. The portal vein was cut into several pieces and incubated for 10 min in low Ca^{2+} (40 μM) physiological solution, then 0.8 mg/ml collagenase, 0.25 mg/ml pronase E, and 1 mg/ml bovine serum albumin were added at 37°C for 20 min. After this time, the solution was removed and the pieces of vein were incubated again in a fresh enzyme solution at 37°C for 20 min. Tissues were then placed in enzyme-free solution and triturated using a fire-polished Pasteur pipette to release cells. Cells were stored on glass coverslips at 4°C in physiological solution containing 0.8 mM Ca^{2+} and used on the same day.

Cells were loaded by incubation in physiological solution containing 1 μM Fura-2-acetoxymethylester for 20 min at room temperature. These cells were washed and allowed to cleave the dye to the active Fura-2 compound for at least 1 h. Fura-2 loading was usually uniform over the cytoplasm, and compartmentalization of the dye was never observed. Measurement

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of intracellular Ca^{2+} concentration was carried out by the dual-wavelength fluorescence method, as previously described [1]. Briefly, Fura-2-loaded cells were mounted in a perfusion chamber and placed on the stage of an inverted microscope (Nikon Diaphot, Tokyo, Japan). A single cell was alternately excited with UV light of 340 nm and 380 nm through a 100 × oil-immersion objective (Nikon, 1.3 NA), and emitted fluorescent light from the Ca^{2+}-sensitive dye was collected through a 510 nm long-pass filter with a Charge-Coupled Device camera (Hamamatsu Photonics, Hamamatsu City, Japan). The signal was processed (Hamamatsu Photonics DVS 3000) by correcting each fluorescence image for background fluorescence and calculating 340/380 nm fluorescence ratios on a pixel-by-pixel basis. Averaged frames were usually collected at each wavelength from a single cell every 0.5 sec. $[Ca^{2+}]_i$ was calculated from mean ratios using a calibration for Fura-2 determined in loaded cells. Some experiments were carried out in the presence of 1 μM oxodipine (a light-stable dihydropyridine derivative) in order to inhibit voltage-dependent Ca^{2+} channels. All measurements were made at 25 ± 1°C.

Voltage-clamp and membrane current recordings were made with a standard patch-clamp technique [6] using a List EPC-7 patch-clamp amplifier (Darmstadt-Eberstadt, Germany). Whole-cell membrane currents were measured with the perforated-patch method [7]. In order to obtain a perforated patch, nystatin (80–100 μg/ml) was present in the patch pipette solution. Patch pipettes had resistances of 1-4 Mω. Membrane potential and current records were stored and analyzed using an IBM-PC computer (P-clamp system, Axon, Foster City, CA).

The normal physiological solution contained (in mM) : 130 NaCl, 5.6 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 11 glucose, 10 HEPES, pH 7.4 with NaOH. The basic pipette solution contained (in mM) : 130 CsCl, 10 HEPES, pH 7.3 with CsOH. For the recordings of calcium channel current, 5 mM $BaCl_2$ was substituted for $CaCl_2$ in the reference solution, and CsCl was used instead of KCl in the pipette and external solutions to block outward potassium currents. In addition, 5 mM EGTA, 5 mM Na_2ATP, 1 mM $MgCl_2$ were added to the basic pipette solution. α_1- and α_2-Adrenoceptor agonists were applied to the recorded cell by pressure ejection from a glass pipette for the period indicated on the records. Before each experiment a fast application of physiological solution was tested and cells with movement artefacts were excluded.

Collagenase was obtained from Worthington (Freehold, NJ); pronase (type E), bovine serum albumin, norepinephrine, clonidine, rauwolscine, prazosin and nystatin were from Sigma (St Louis, MO). Caffeine was from Merck (Nogent sur Marne, France). 1-(6-((17β-3-methoxystra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione (U73122), 1-(6-((17β-3methoxystra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-pyrrolidine-dione (U73343) and tricyclodecan-9-yl xanthogenate K (D609) were from Biomol (Plymouth Meeting, USA). Oxodipine was a gift from Dr Galiano (IQB, Madrid, Spain).

The results are expressed as means ± S.E.M. Significance was tested by means of Student's t test. P values of <0.05 were considered as significant. Inhibition and concentration-response curves were analyzed by a nonlinear least-square fitting program, according to models involving one- or two-binding sites.

RESULTS

In order to verify that U73122 inhibits phosphatidylinositol-phospholipase C in vascular myocytes, experiments were performed on the norepinephrine-induced Ca^{2+} release from the intracellular stores (in the presence of 10 nM rauwolscine and 1 μM oxodipine). The norepinephrine-induced Ca^{2+} response was concentration dependently inhibited by U73122 with an estimated concentration producing half-maximal inhibition (IC_{50}) of 0.25 μM (Fig. 1A). In contrast, U73343, which had no effect on phospholipase C, did not inhibit the noradrenaline-induced Ca^{2+} release up to 1 μM. U73122 and U73343 had no direct inhibitory effect on the intracellular Ca^{2+} stores as the transient increase in $[Ca^{2+}]_i$ was obtained with 10 mM caffeine in the continuous presence of 1-10 μM of these compounds (Fig. 1B). It has to be noted that the caffeine-induced Ca^{2+} response (control : 282 ± 11 nM, n = 12) was not affected by application of 10 μM U73122 (270 ± 13 nM, n = 12). The degree of inhibition by U73122 of the norepinephrine-induced Ca^{2+} release was not modified by varying the time the cells were exposed to U73122 from 3 min to 15 min. However, U73122 and U73343 inhibited the L-type Ca^{2+} channel current in a concentration-dependent manner with estimated IC_{50} values of 0.1 μM and 0.7 μM, respectively (Fig. 1C). These results suggest that, in addition to inhibition of phosphatidylinositol-phospholipase C, U73122 blocks the L-type Ca^{2+} channel of vascular myocytes.

We also examined the ability of D609 to block phosphatidylcholine-phospholipase C and secondarily to inhibit the activation of PKC by preventing the generation of DAG. When externally applied for 20-30 min, D609 had no direct effect on the L-type Ca^{2+} channel current at concentrations up to 100 μM (Fig. 2A). The clonidine-induced stimulation of Ca^{2+} channel current (in the presence of 10 nM prazosin) was concentration dependently inhibited by D609. As illustrated in Fig. 2B, application of 10 μM D609 for 15 min suppressed the clonidine-induced stimulation of

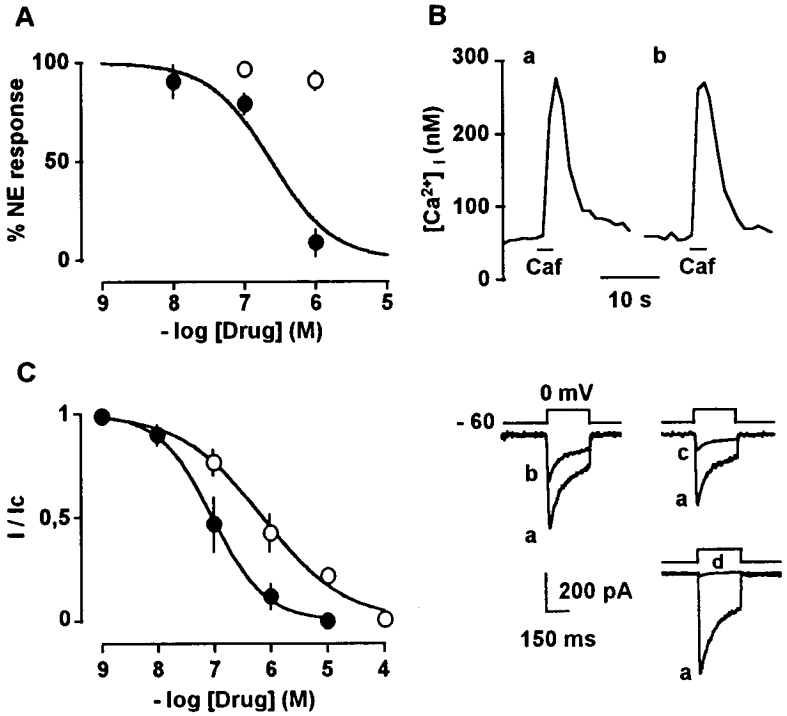

FIG. 1. Effects of Ca^{2+} from intracellular stores in single myocytes from rat portal vein. (A) Inhibition curves of the norepinephrine-induced release of stored Ca^{2+} by U73122 (●) and U73343 (○). $[Ca^{2+}]_i$ values are expressed as a percentage of the response obtained with 10 μM norepinephrine (NE). Each point represents the mean ± S.E.M. for 5–13 cells. External solution contained 2 mM Ca^{2+}, 10 nM rauwolscine (to inhibit α_2-adrenoceptors) and 1 μM oxodipine (to block voltage-dependent L-type Ca^{2+} channels). (B) Effect of U73122 on the caffeine-induced release of stored Ca^{2+}. a: control; b: in the presence of 10 μM U73122 for 15 min. Similar results were obtained in 10 cells. (C) Ca^{2+} channel current was evoked by a deporalization to 0 mV from a holding potential of −60 mV with the nystatin-perforated patch method. Inhibition curves induced by applications of U73122 (●) and U73343 (○). Current values are expressed as a fraction of their control values (I/Ic). Each point represents the mean ± S.E.M. for 4-10 cells. External solution contained 5 mM Ba^{2+}. Inset, current traces from three different cells obtained in control (a) and in the presence of 0.1 μM (b), 1 μM (c) and 10 μM (d) U73122.

Ca^{2+} channel current (control : 22 ± 3%, n = 3; in the presence of D609: 3 ± 2%, n = 5). However, the norepinephrine-induced Ca^{2+} response (in the presence of 10 nM rauwolscine and 1 μM oxodipine) was concentration dependently inhibited by D609, as illustrated in Fig. 2C. It has to be noted that the D609 pretreatment induced a significant enhancement in basal $[Ca^{2+}]_i$ which increased from 55 ± 15 nM (n = 12) in control conditions to 115 ± 45 nM (n = 9) in the presence of 10 μM D609. In addition, D609 inhibited the caffeine-induced Ca^{2+} response which was decreased by 35 ± 5% (n = 5) and 95 ± 5% (n = 7) with 10 μM and 100 μM D609, respectively (Fig. 2D). These results suggest that, in addition to inhibition of phosphatidylcholine-phospholipase C, D609 blocks the intracellular Ca^{2+} stores that can be mobilized by caffeine or norepinephrine.

DISCUSSION

The conclusion that U73122 inhibits phosphatidylinositol-phospholipase C in vascular myocytes is consistent with the observation that this drug inhibits the norepinephrine-induced release of Ca^{2+} from the intracellular stores, which has been demonstrated to be dependent on both activation of α_{1A}-adrenoceptors and inositol phosphate formation (1). This effect appears to be specific since the

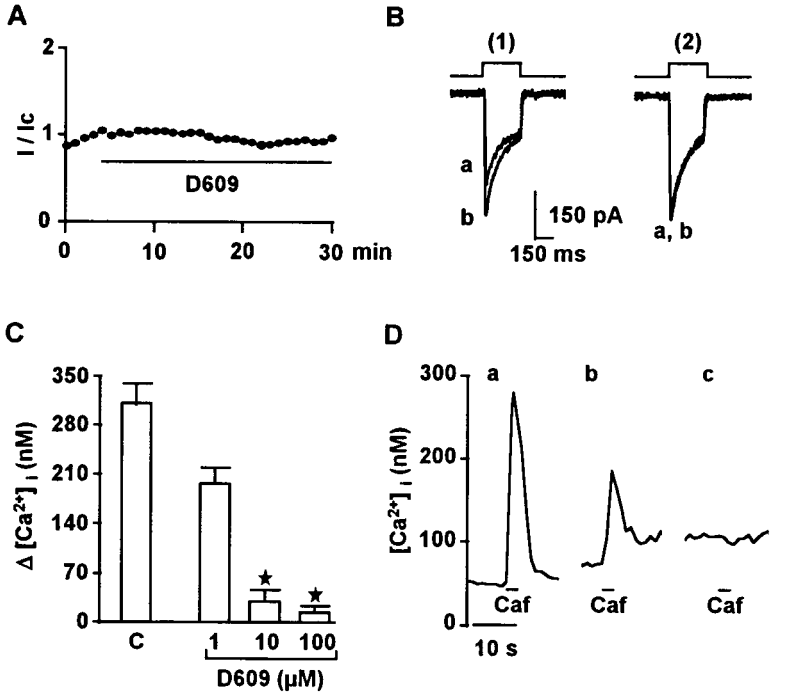

FIG. 2. Effect of D609 on Ca^{2+} channel current, Ca^{2+} release from intracellular stores and clonidine-induced stimulation of Ca^{2+} channels in single myocytes from rat portal vein. (A) Ca^{2+} channel current was evoked by a depolarization to O mV from a holding potential of −60 mV with the nystatin-perforated patch method. Time course of the peak current during the application of 100 μM D609. Currents are expressed as a fraction of their control values (I/Ic). External solution contained 5 mM Ba^{2+}. (B) Effect of D609 on the clonidine-induced stimulation of Ca^{2+} channel current. Ca^{2+} channel currents evoked by a depolarization to 0 mV from a holding potential of −60 mV in the presence of 10 nM prazosin (to inhibit α_1-adrenoceptors) in control conditions (1) and after application of 100 μM D609 for 15 min (2) before (a) and during the application of 10 μM clonidine (b). External solution contained 5 mM Ba^{2+}. (C) Inhibition of the norepinephrine-induced release of stored Ca^{2+} by increasing concentrations of D609. Each point represents the mean ± S.E.M. for 7-12 cells. External solution contained 2 mM Ca^{2+}, 10 nM rauwolscine and 1 μM oxodipine. (D) Effect of D609 on the caffeine-induced release of stored Ca^{2+}. a: control; in the presence of 10 μM D609 (b) or 100 μM D609 (c). Similar results were obtained in 5-7 cells.

inactive analog, U73343, has no effect on the norepinephrine-induced release of stored Ca^{2+}. However, these compounds have other sites of action : (1) U73122 inhibits the voltage-dependent L-type Ca^{2+} channel in a concentration-dependent manner with an efficiency similar to that inhibiting phospholipase C, but has no direct effect on the intracellular Ca^{2+} stores which evoke caffeine-induced Ca^{2+} responses similar to those obtained in control conditions ; (2) U73343, which is not an inhibitor of phospholipase C, inhibits voltage-dependent Ca^{2+} channels, but has not effect on the norepinephrine- and caffeine-induced Ca^{2+} responses. This suggests that the mechanism of inhibition of Ca^{2+} channels by U73122 differs from that for inhibition of phospholipase C action. It has been also reported that U73122 may inhibit store-operated Ca^{2+} influx [8], phosphatidylinositol kinase and phosphatidylinositol 4-phosphate kinase [9] and phospholipase D [10].

As D609 has no direct effect on voltage-dependent L-type Ca^{2+} channels, its action on phosphatidylcholine-phospholipase C may be revealed by studying the transduction pathways that involve Ca^{2+} channel modulation. Our results show that D609 inhibits the α_{2A}-adrenoceptor-induced stimulation of Ca^{2+} channels, which has been reported to be independent of phosphatidylinositol hydrolysis and $InsP_3$ production [4, 11]. However, D609 inhibits in a concentration-dependent manner both the norepinephrine- and the caffeine-induced Ca^{2+} release indicating that this drug interferes with the intracellular stores in a nonspecific manner.

Previous data have demonstrated that the α_{2A}-adrenoceptor-evoked stimulation of Ca^{2+} channels is related to activation of PKC in response to increased DAG formation [11]. DAG production can be mediated either by phospholipase C or phospholipase D [12]. The action of phospholipase C would result in the formation of DAG directly. The action of phospholipase D would result in the formation of phosphatidic acid and choline ; the hydrolysis of phosphatidic acid by phosphatases would then lead to the subsequent formation of DAG. Our results suggest, for the first time in vascular myocytes, that α_{2A}-adrenoceptors activate a phosphatidylcholine-phospholipase C to generate DAG and the subsequent activation of L-type Ca^{2+} channels.

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REFERENCES

1. Leprêtre, N., Mironneau, J., Arnaudeau, S., Tanfin, Z., Harbon, S., Guillon, G., and Ibarrondo, J. (1994) *J. Pharmacol. Exp. Ther.* **268,** 167–174.
2. Mironneau, C., Rakotoarisoa, L., Sayet, I., and Mironneau, J. (1991) *Eur. J. Pharmacol. Mol. Pharmacol. Section* **208,** 223–230.
3. Gutierrez, L. M., Zhao, X. L., and Hosey, M. M. (1994) *Biochem. Biophys. Res. Comm.* **202,** 857–865.
4. Leprêtre, N., Mironneau, J., and Morel, J. L. (1994) *J. Biol. Chem.* **269,** 29546–29552.
5. Leprêtre, N., Ibarrondo, I., Arnaudeau, S., Morel, J-L., Guillon, G., and Mironneau, J. (1995) *Pflügers Arch.* **430,** 590–592.
6. Hamill, O. P., Marty, A., Neher, E., Sakmann, B., and Sigworth, F. J. (1981) *Pflügers Arch.* **391,** 85–100.
7. Horn, R., and Marty, A. (1988) *J. Gen. Physiol.* **92,** 145–159.
8. Berven, L. A., and Barritt, G. J. (1995) *Biochem. Pharmacol.* **49,** 1373–1379.
9. Vickers, J. D. (1993) *J. Pharmacol. Exp. Ther.* **266,** 1156–1163.
10. Gratas, C., and Powis, G. (1993) *Anticancer Res.* **13,** 1239–1244.
11. Leprêtre, N., and Mironneau, J. (1994) *Pflügers Arch.* **429,** 253–261.
12. Exton, J. H. (1990) *J. Biol. Chem.* **264,** 1–4.