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Original Research

Effect of U-73122 oo calciwn channel activity in porcine myometrial cells and pancreatic beta-cellline RINm5F

LI Si-Ben ffiN Ter-Hsin ZHU-GE Rong-Hua Walter H Hslf
(Department of Veterinary Physiology and Phamacology Iowa State Universi Ames IA 51 11USA)

KEY WORDS Bay-K-8644; calcium; calcium channels; Fura-2; islets of Langerhans; myometrium; patch-clamp techniques; phospholipase C; U-73122; U-73343

AIM: To study the effi t of U-73122 putative phospholipase C (PLC) bi on cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and voltage pendent calcium channels (V. MEODS: To record whole cell calcium current (I_{Ca}) by perfora clamp and to m sure cytosolic ee calcium concentration ($[Ca^{2+}]_i$) Fura-2. SUL : U-73122 inhibited α-inced and Bay-K- 44-induced increase in $[Ca^{2+}]_i$ dose-dependently. U-73122 creased rnarlcedly depolion-induced I_{Ca} both in RINm5F and porcine myometrial cells in a concentration-dependent manner. The effect of U-73122 was more p t in porcine myometrial cells than that in RINm5F cells. U-73343 (an analog of U-73122) at does not inhibit PLC also redu e I_{Ca} by 5 %. CONCLUSION: U-73122 ibited VDCC and +linbo RINm5F and porcine myometrial cells.

Activation phospholipase C (PLC) by hormones and neurotransmitters is an important intracellular signal transduction pa way. The downstream of PLC-mediated signals plays an important role in various llular functions. U-73122 1-[6-[[17-me oxyestra 35(10)- ttien -17-yl J amino]-hexyl J-IH-pyrrole-2 5-dion putative inhibitor of PLC has been applied in the studies of the receptor-mediated PLC-dependent pro ss in many different cell types such as platelets and neutrophils[1], Chinese hamster ov y cells pancreatic acinar cells insulin-secreting cell lines RINm5F[2] and TC3[3] pancreatic AR42J cells a neuroblastoma cell line neuronal cells pituitary gonadotrophs rat cosωchondral chondrocyt and porcine myome al cells[4]

U-73122 has additional actions at are independent of the inhibition of PLC activity. . For example U-73122 stimulates e release of Ca^{2+} from endoplasmic reticulum (ER) of the rat liver ugh inhibition of Ca^{2+} uptake by Ca^{2+}-Ä e[5] U-73122 bl ks KCl-induced (50 mmol.L-1) Ca^{2+} influx in NG108-15 cells but not in dorsal 1 t ganglion nens. U-73122 also inhibits e Ca^{2+} influx induced by psigargin a bl ker of ER Ca^{2+}-ATPase in mouse fibroblasts[6].

We have used U-73122 as a PLC inhibitor in porcine myometrial cells to study the mechanisms of oxytocin receptors[4] and in RINm5F cells to study the mechanisms of muscarinic and arginine-vasopressin receptors[2]. In is study we explored the effect of U-73122 on Ca^{2+} influx via VDCC in RINm5F cells and e native porcine myometrial cells to demons te the effect of U-73122 on these endocrine cells and smooth muscle cells. le major VDCC is e L-type channel in e pancreatic cells[7] and sm th muscle cells. Besides we used one of L-type VDCC agonist Bay-K-8644[8] 14-dihydro-26-dime yl-5-4-[2-(luoro-methyl)-phenyl J-3-p dinecarboxylic acid and an analog of 73122 73343[9] 1-[6-[[17methoxy -135(0)- en-17-yl J-amino J-hexyl J-25-pyrrolidine-dione which should not effect on PLC.

MATERIALS AND METHODS

CeU culture RINm5F cells were maintained in Roswell Park Memorial Institute medium 1640 containing 10 % fetal bovine serum and aera d with 5 % C/95 % r at 37 t:[2] All experiments were performed wi cells from passages 45-54.

of Medical Science Ming Railv. Medical College
Now in Institute . m: 86-25-330-151 ext 2592
Nanjing 210009, Prof Walter H HSU
Correspondence 1-26 A epted 1997-05-26
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premtion of myometrial cells Porcine uteri were obtained from a local slaugh r house; le myomeal cells from pregnant sows in late pre ancy (d 107 - 112) were isolated(IO).

R ordi ngs of 1 ca The perforated pa _clamp(U) was ωed to record whole cell lea with an Axopatch lD voltage-claIlp aIlplifier (Axon InstrumentsFoster City CA). Patch pipe s (3 – 5 Mn) were made of dispo able glass pipes (VWR Scientific West Chester PA) by a two-stage pull and t polished. The liquid junction was nullified with an offset circuit befo the formation of gigaseals. Linear resistance d capacitative currents were el ctronically compensa d by the *P/N* prot ol. Currents were low-pωs filtered at 1 Iz. The data were acquired and analyzed using an ffiM computer with the pClaIlp software (Axon Instruments). The pa h pipette was filled with a solution containing L-glutamic acid 12 mmol-L-1 CsO H 130 m mol- L-1 aIlphotericin B 250 mg - L-1 HEPE5 1 0 mmo l-L-1 (pH 7.2). The bath ωlution contained tetraethyl-ammonium chloride 140 mol- L-1 4-aIlinopyridine 5 mmol-L-1CaCl22 mmol-L-1 dex ωe 1.67 mol - L-1 KO 5.4 mmol- L-1 and Hl0 mmol-L-1. U 122 was applied t ebasolution directly and all experiments we rformed at r m tem rarure. In RJNm5F cells the concen tions of Ul22 were acrual (but not cumulative). In myometrial cells Je αxlCentrations of U-73122 w cumulative due to technical difficulty in perforating patch-cl np eximents in >reine Ils.

Measurement of [ea2+]i in RINm5F cells To m[+]; in all cell suspensions 30 x 1(/ cells were loaded with Fura-2 acetoxymethylester (AM) 2μnol-L-1 in KRB at 37 't for 30 min. ηle load cells were washed and kept at 24't until use. Cells were resuspended at a concentration of 10'1 - L-1 Id a 1.5 mL aliquot was used for [+] i determination at 24't. The 3401 nm fluorescence ratiωwere monitored in an SLM-8000 fluoresce e s trl photome(SLMU anaIL). The [Ci!+]; was calibrated after the celllysis(12). When nee dU-73122 was given 1 s befo Je adminis tion of KO or Bay-K- 4.

S tistical analysis Results were !alyzed by paired and unpaire *t* test.

Materials Fura 2-AM (Molecular 00Eugene OR) U-73122 and U-7 43 (Biomol Research Laboratcnou Meeting PA) Bay-K-4 (Research Bi hemicals IncNatick M A). Fura 2-AM was dissolved in M SO Bay-K- 44 was dissolved in 95 % e anol. U-73122 and U-7 43 were dissolved in chloroform and dispen d in aliquots (10 nmoI/20 μL) and thert the chloroform was evaporated under a s am of N2. s left a dry film of U-73122 or U-73343 in the container which was stored in a desiccator. ηle dry film was dissolved in M SO immediately before us Wh en ethanol or MSO was used as a solvent the final concentration of the organic solvent was 1: 10 in aqueous solution.

RESULTS

Eect of U-73122 on KCl- and Bay-K-864 induced increase in [Ca2+]i of RINm5F cells The [Ca2+] rise after KCl (15 mmol - L-1) ωually reached a m imwn by 25 s and stayed at the plu level for 3 – 4 min. Pretreatment wiU-73122 (1-8 μmol - L-1) for 1 sinlbited KCl (15 nimol- L-l). induced incr eases in [ea2+]i do de pe nde ntly. U-731228 μmol - L-1 inhibited KCl (15 m mol- L-l). induced increase in [ea2+]i by 46 % (Tab 1).

Tab 1_ Eff t of U-73122 on KCl- and Bay. (4 induced peak [ecr+]; in RINm5F cells_ U.73122 given 1 s before KCl (15 mmol- L-1) or Bay 4(1 -L-1)_ *n*=4 s_ "1'>0_05" P<O.

U-73122 /μmol-L-1	KO-induced ι[C]/nmol-L-1	Bay-K- 4-induced .c2+]/nd-L-1
[illegible]	47.2 : t1.7	25.1: t1.0
	46.7 :t 1.0'	19.6:t 0.6b
	37.2 2.0b	16.2:t 0.8b
	31.2:f:13b	14.1:t 1.0b
	25.3:f: 2.0b	10.9 01'

When Ba y-K- 44 (1 μmol-L-1) Wωapplied R m5F 11sit caused a rapid increase in [ar+ li which was sustained for 3 – 4 min. le effect of B K-8ω4 (1 μmol - L-1) was abolished by the L. VDCC bl ker nimodipine (1 fLmol - L-1). To examine er e effect of U-73122 was sificto PLC we investigated its effect on Bay-K induced increase in [ea2+]i. Pretreatm wi U-73122 (1-8 μmol - L-1) for 1 s inhibi K 4 (1 μmol- L-1)-induced increase in [C dose-dependently (Fig 1B). U-73122 8μnoi-L-l inhibited Bay-K-8644 (1 μmol- L-1)-induced m in [ω2+ 1 by 57 %.

Inhibition by U-73122 of depolariza induced 1 ca in RINm5F cells At the holdi potential (HP) of – 70 mV curren were elici 3 rns depolarization steps from – 30 mV i + 50 mV. The maximal 1Ca was evoked by :polarizing pulse to + 20 mV. U-73122 (2 and μmol - L-1) reduced the ak 1Ca' but did not shift me mbrane potential value of the initial or akαm (Fig l).

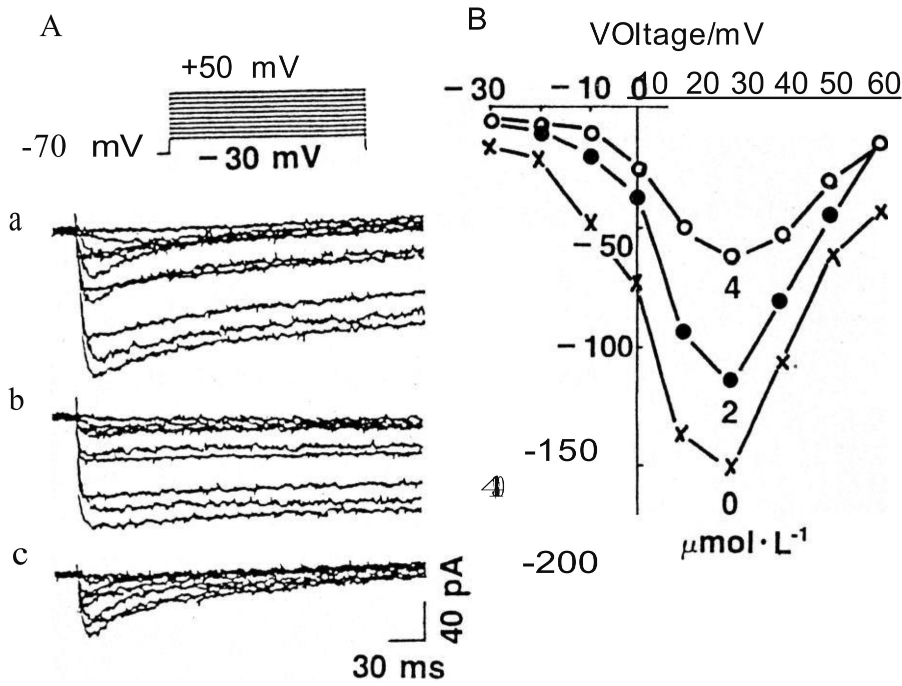

Fig 1. Inhibition by U-73122 of depolarization-induced lωin RINm5F ceUs. Currents w e elici dby3 ms depolarization steps om −30 mV to +50 mV at tbe holding potential of − 70 mV. Aαm t acings ob ined under control solution (a) and in tbe pre nce of U-73122 (b 2 μmol.L-1 and c 4 μmol.L-1) B corre)()nding I-V relationship. Da shown are repr en tiVI of 4 expe nents.

The control 1 ak I_{Ca} was established by depolarizmg pulses from a HP of − 70 mV to + 20 mV. This depolarizing s p was applied every 10 s which elicited stable I_{Ca}. U-73122 (8 μmol.L 1) abolished the I_{Ca} M 2 mi:n of administration (Fig 2A). The effect of U-73122 on I_{Ca} was reversed by a washout and a second application of U-73122 s 1 produ dan apparent inhibition of I_{Ca}. The I_{Ca} evoked by depolarization in RINm5F cells was inhibited by U-73122 (1 − 8 μmol . L -1) m a dose-dependent manner (Fig 2C). However U-73343 (8 μmol L-1 an analog of U-73122 at does not inhibit PLC, ue I_{Ca} by 50 % ± 4 % (*n* = 4) (Fig 2B).

Inhibition by U-73122 of depolarization-induced I_{Ca} in porcine myome cells At the HP of − 50 mV currents were elicited by 3 ms depolarization steps from − 30 mV to + ω mV. Like RINm5F cells em imum I_{Ca} was evoked by the depolarizing pulse ω + 20 mV. U-73122 (30 and 50 nmol · L-1) reduced ak I_{Ca} • but did not shift the membrane potential value of the initial and peak current (Fig 3).

We selected 6 cells from 3 different tissu to study the cumulative dose-response ω U-73122. le ak I_{Ca} was evoked by a 3 ms depolarization s p from an HP of − 50 mV ω +20 mV. s depolarizing s p was plied every 20 s. Because the inhibitory effect of each dose of U-73122 reached a stable level within 1 s each dose of U-73122 was administered every 1 s. U-73122 caused a concentration-dependent inhibition of I_{Ca}. At the highest ncentration of U-73122 studied (1 μmol • L-1) I_{Ca} was nearly a (Fig 4).

DISCUSSION

U-73122 has k :n ed to study e PLC-media physiological events and mechanisms underlymg these events(13). Activation of PLC leads to mcreased mositol 145-trisphospha (IP3) fonnation which m tum promotes e release of Ca^{2+} from e ER and Ca^{2+} influx through opening of VOCC and :ptor-opera d Ca^{2+} ch mels(14) U-73122 spec lly inhibits PLC-mdu d Ca^{2+} rel se and influx(2 5) • However U-73122 ndir tly

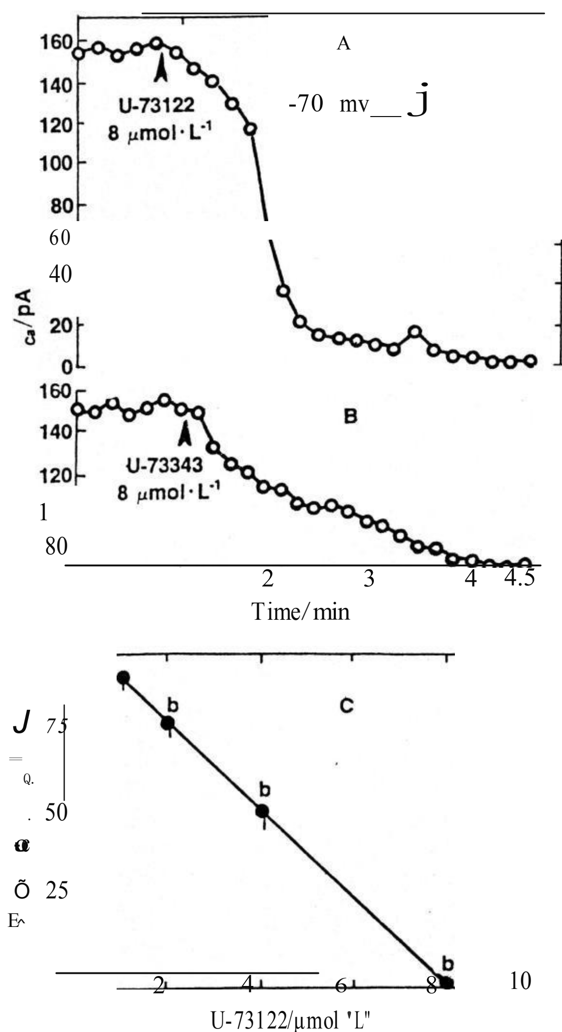

Fig2. I t ibition by U-73122 of 1 generated by one-step depolarizations in RINm5F cells. Cun were elicited oma holng pottial of − 70 mV to + 20 mV every 10 s. A ne course of inhibition by U-73122. Bte course of ibition by U-73343. Data showΠ in A and B are repn e of 4 e eriments. Cdose-response to U-73122 (1 − 8 101 • L-1). Maximmn k lea was recorded 10 min aft application of U-73122 wh e eff tr ched a s ble level. n =4 s . bp < 0 .05 vs control .

inhibit ea2+ influx which is independent of PLC activation. effect of U-73122 has en demonstrated in mouse fibroblasts where U-73122 redu d the ea2 + influx induced by apsigargin an inhibitor of e ER ea2 + -ATPaseand in differentiated NG1OO-15 1 lswhere U-73122 blocks mpletely e KC1-induced ea2 + inflUX(4). KCl increases ea2 + influx by depolar ing the membrane potentialand opening V IC. Administering a VDCC agonist such as Bay-K-8644 is another method of opening VOCC. In the present study we showed M U-73122 at effective concentrations for inhibiting PLC decre ed KC1- and Bay-K-8644-indu d ea2 + influx in RINm5F ce1ls. In addition U-73122 at th concentrations inhibited d0 lation-induced 1 ! in RINm5F ce1ls and porcine myo metria lω Us U-73343 the inactive analog of U-73122 on P also inhibited 1ca of RINm5F ce1ls but was less effective than U-73122. The results sugges e inhibiωry effect of U-73122 on V ::C is independent of the inhibition of PLC. le difference between U-73122 and U-73343 could be due ωthe slight difference in chemical strucωm

U-73122 may have different sensitivity different ce1l types with regard to its inhibitory effi on ea2 + influxbecause it blocks KC1-inducOO ea2+ influx in NG1OO-15 cells but not in dorsal root ganglion neurons(4). our results further sues porcine myometrial ce1ls were more sensitiveω U-73122 inhibitory eect on VOCC than RINm5F 11s. These results are interesting becau : 1) dose responses of U-73122 in inhibiting lea and PLC. mediated ea2 + rel parallels each 0 :r in 00th pòrcine myome al ce1ls and RINml1sand 2) U-73122 is more effective in inhibiting bo e land PLC-mediated Ca2 + rel se in porcine myometrial ωlls than RINm5F 11S(2 35) . Howeverit is not clear why U-73122 is more effective in porcine myomd ce1ls than RINm5F ce1ls.

h e present study U-7312was more potent in inhibiting Ica than KC1- or Bay-K-8644-in increase m [Ca2+]r·IB is mbe attribl ω fact at patch clamp experiments were perfomM single 11swhereas e experiments on [ea2 + 1were performed in a cluster of cells. le clusterωl!d afle uptake of the drug into individual cells.

inhibitory effect of U-73122 on V ::C r be attributed to a possible mech m. U-73122 is antinos riod which is lipophilic and readily diffuses rough the plásrna membrane or adheres to e pla membrane thereby potentially changing co ! ormation and/or activity of VDCC. We found the binding of U-73122 to cells is

an ses na the also reversible

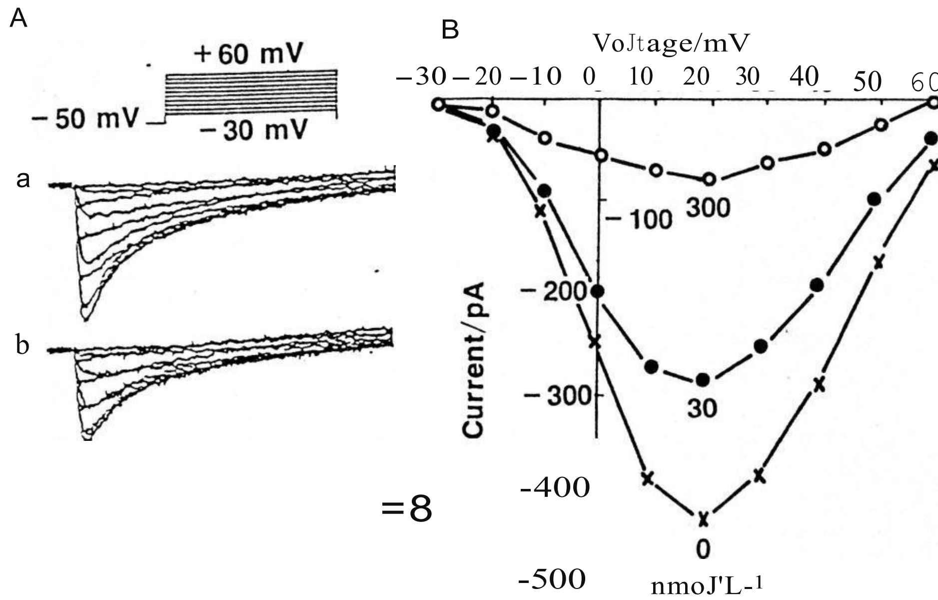

Fig 3. Inhibition of U-73122 of depolarization-induced lea in porcine myometrial cells. Currents were elicited by 300 depolarization steps from −30 mV to +60 mV at the holding potential of −50 mV. A current tracings obtained under control solution (a) and in the presence of U-73122 (b30 nmol' L^{-1} and c3 nmol' L^{-1}). B, corresponding I-V relationship.

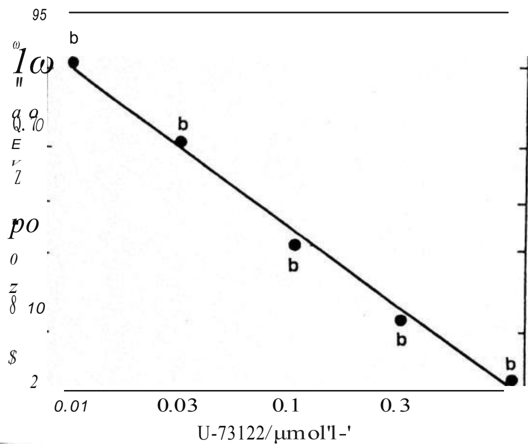

Fig 4. Inhibition of lea by U-73122 in porcine myometrial cells. Currents were elicited from a holding potential of −50 mV to +20 mV. U-73122 was administered in a cumulative manner at the interval of 300 s. n=8. $^bP<0.05$ vs control.

its effect on Ica can be washed out by exchange of medium. Further studies are needed to elucidate the mechanisms underlying the inhibitory effect of U-73122 on VDCC.

U-73122 has additional effects that independent of PLC inhibition. In rat liver microsomes(6) and rabbit pancreatic acinar cells(15) U-73122 releases Ca^{2+} through inhibition of Ca^{2+}-ATPase on the ER. However only high concentrations of U-73122 (≥10 μmol'L^{-1}) increase Ca^{2+} release noticeably. These concentrations exceed the dose range for inhibiting PLC-mediated Ca^{2+} release(6,15)

In conclusion U-73122 inhibits VDCC in RINm5F cells and porcine myometrial cells. It is likely that the inhibition of VDCC by U-73122 may not be attributed to its inhibitory effect on PLC. Further studies are needed to determine whether the inhibitory effect of U-73122 on PLC contributes to its inhibition of VDCC. Because the concentration range for producing the inhibitory effect on VDCC is similar to that for inhibition of PLC activation caution must be exercised in interpreting the results from studies of PLC-mediated changes in Ca^{2+} channel activity.

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U-73122 P
RINm5F

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(Department ιVeterinary Physiology and PhannacoÚJgyIowa State UniversityAmes IA 11USA)

Bay-K- 44; ; ; Fura ;
; ; C; U-73122i

U-73343

: U-73122

. : Fura-2

: U-73122 R m5F

KCl Bay-K-44

. U-73122

RINmSF C

U-73122 U-73343 50 %.

: U-73122 RINmSF

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