Vol. 165, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1989 Pages 416-421

ACTIVATION OF PROTEIN KINASE C ASSISTS INSULIN PRODUCING CELLS IN RECOVERY FROM RAISED CYTOPLASMIC Ca^{2+} BY STIMULATING Ca^{2+} EFFLUX

Per-Olof Berggren*, Per Arkhammar and Thomas Nilsson

Department of Endocrinology, Karolinska Institute, Karolinska Hospital, Box 60 500, S-104 01 Stockholm, Sweden

Received October 9, 1989

The effects of protein kinase C (PKC) activation on the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) were studied in clonal insulin-producing RINm5F cells, using the fluorescent Ca^{2+} indicators quin-2 and fura-2. Both under basal and stimulatory conditions PKC activation lowered $[Ca^{2+}]_i$ in these cells by promoting an active extrusion of Ca^{2+} to the extracellular space. PKC activation therefore assists insulin-producing cells in recovery from raised $[Ca^{2+}]_i$. Such an effect might be part of the signal regulating the insulin secretory process.

Insulin-producing pancreatic β-cells are similar to other cells in demonstrating generation of inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG) subsequent to activation of phospholipase C (for review, see 1). Whereas $InsP_3$ releases intracellularly bound Ca^{2+} (2), DAG exerts its effects through the activation of protein kinase C (PKC) (for review, see 3). Activity of this enzyme has been established in both rat (4) and mouse (5,6) β-cells as well as in insulin-producing tumor cells (7,8). The phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA), in nanomolar concentrations, is assumed to stimulate PKC specifically and has been used extensively as a substitute for DAG (3). Since the original observation that TPA stimulates insulin release (9,10), a number of investigations have indicated a role for PKC in the regulation of insulin secretion. One such role might be to serve as a negative-feedback modulator of stimulated Ca^{2+} influx and thereby assist in recovery from a rise in the free cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$), as we have recently proposed (6). By using the clonal insulin-producing RINm5F cells, we have in the present study addressed more specifically the question of how such a recovery might be regulated.

* To whom correspondence should be addressed.

0006-291X/89 $1.50

MATERIALS AND METHODS

All reagents were of analytical grade and redistilled deionized water was used. Whereas quin-2/acetoxymethylester (quin-2/AM), dimethylsulphoxide (DMSO) and TPA were from Sigma St. Louis, MO, fura-2 free acid (fura-2) was purchased from Calbiochem, La Jolla, CA. D-600 was a gift from Knoll A.G., Ludwigshafen am Rhein, F.R.G. and bovine serum albumin fraction V was obtained from Boehringer Mannheim GmbH, Mannheim, F.R.G.

The clonal insulin-producing RINm5F cells were cultured and cell suspensions were prepared, as previously described (11). The suspensions of RINm5F cells were incubated for at least one hour before any experiments were performed. If not otherwise stated, the medium used for measurements of $[Ca^{2+}]_i$ was a Hepes buffer, pH 7.4, supplemented with 1 mg/ml bovine serum albumin, containing 1.3 mM Ca^{2+} and with Cl^- as the sole anion (12). For studies of permeabilized cells we used a Hepes buffer, pH 7.0 (adjusted with KOH), containing 0.5 mg/ml of bovine serum albumin and in mM: 25 Hepes, 110 KCl, 10 NaCl, 2 KH_2PO_4, 1 $MgCl_2$ and 5 succinate.

RINm5F cells were incubated for 30 min with 2.5 μM quin-2/AM, which gave loadings of 1 - 4 nmol quin-2 per 10^6 cells. The estimations of RINm5F cell numbers were based on the assumption that 1 mg dry weight corresponds to about 4 x 10^6 cells (13). $[Ca^{2+}]_i$ was measured and calibrated as previously described (14). Neither DMSO, chosen as the vehicle for diluting TPA, nor TPA affected cellular autofluorescence, as checked in separate experiments using cells not loaded with quin-2 (data not shown). In the Ca^{2+} efflux measurements, 2 μM fura-2 and 4 μM EGTA were added to a Ca^{2+} deficient medium, which gave Ca^{2+} concentrations in the medium of about 100 - 200 nM. DMSO or TPA was added to a suspension of approximately 4 x 10^6 cells and the excitation fluorescence ratio 340/380 nm was recorded at 37°C every minute for a total of 10 minutes. The emission wavelength was 510 nm. Values of extracellular Ca^{2+} concentration were calculated according to 15, using a K_d for Ca^{2+}-fura-2 of 224 nM.

Cells were permeabilized by exposure to high-voltage discharges (five pulses of 5 kV/cm), after being washed twice in cold permeabilization buffer. Subsequent to permeabilization, the cells were centrifuged and the resulting pellet was suspended in 20 μl of the above medium supplemented with 2 mM MgATP and an ATP-regenerating system consisting of 15 mM phosphocreatine and 20 units/ml creatine kinase. The cell suspension was stirred magnetically and the Ca^{2+} concentration in the medium was measured at room temperature with a Ca^{2+}-selective mini-electrode constructed and calibrated essentially as in reference 16. All traces shown are typical for experiments repeated with at least three different cell preparations.

RESULTS AND DISCUSSION

Recently we found that activation of PKC by TPA in normal pancreatic β-cells lowered the increased levels of $[Ca^{2+}]_i$ evoked by either glucose, sulfonylurea or high concentrations of K^+ (11). A similar effect of 10 nM TPA is obtained in clonal insulin-producing RINm5F cells stimulated with a high concentration of K^+ (Figure 1B), an effect that has previously been demonstrated also in other clones of RINm5F cells (17). As depicted in Figure 1 A, the cells were not affected by the addition of 0.2 % DMSO, which was the final concentration in the actual TPA experiments.

The RINm5F cells represent an interesting model system when investigating the mechanisms regulating insulin release. In these cells TPA induced a minor depolarization, a lowering in basal $[Ca^{2+}]_i$ and stimulation of insulin release

(11). The fact that the phorbol ester did not evoke similar effects on membrane potential and $[Ca^{2+}]_i$ in normal pancreatic β-cells (10), is not likely to be accounted for by a difference in either PKC activity or distribution, as suggested from PKC activity measurements (Welsh, M., Welsh, N. and Berggren, P.-O., unpublished observations). Hence, an explanation to this phenomenon can at the moment just be of a speculatory nature, but might simply be due to PKC having a greater influence on the regulation of $[Ca^{2+}]_i$ in insulin-producing tumor cells compared to normal β-cells. Alternatively, the results may reflect differences in the sensitivity of RINm5F cell and normal β-cell C kinases to TPA. When discussing such possible differences, it should be remembered that also RINm5F cells kept in various laboratories differ. It was for example reported that TPA induced a depolarization and a marked increase in $[Ca^{2+}]_i$ under basal conditions, an effect that was inhibited by blockers of voltage-activated Ca^{2+} channels (18). Although similar results were demonstrated in a recent paper (19), it was previously shown that TPA-induced PKC activation was without effect on basal $[Ca^{2+}]_i$ (17). Nevertheless, in our RINm5F cells the phorbol ester even induced a lowering in $[Ca^{2+}]_i$ when basal $[Ca^{2+}]_i$ was further reduced by omission of extracellular Ca^{2+} (Figure 2D).

Previous studies have demonstrated that TPA failed to affect either glucose- or tolbutamide-stimulated uptake of $^{45}Ca^{2+}$ in normal β-cells (10,20). Moreover,

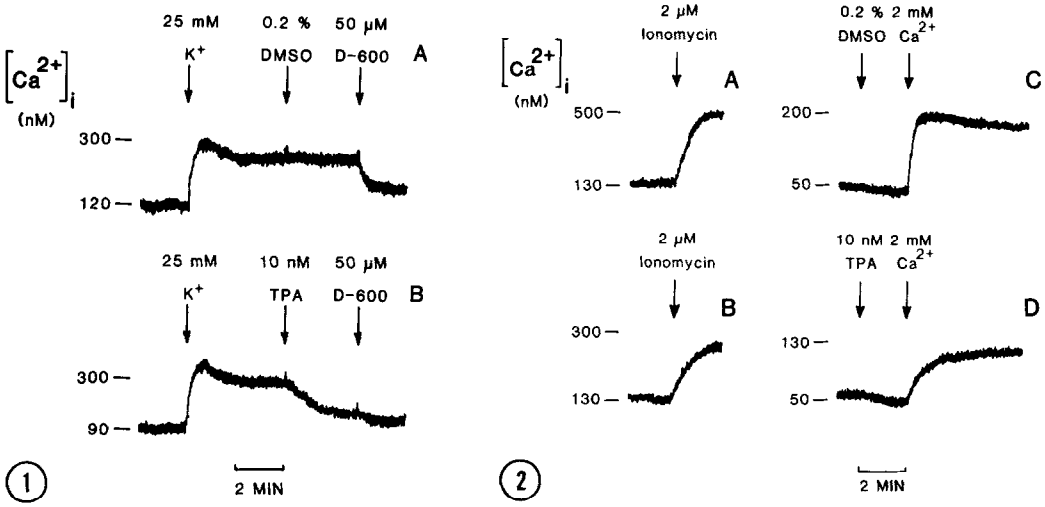

Fig. 1. Effects of DMSO (A), TPA (B) and D-600 on the K^+-induced increase in $[Ca^{2+}]_i$. The actual concentration of the various compounds given is indicated.

Fig. 2. Effects of DMSO and TPA on the increase in $[Ca^{2+}]_i$ evoked by ionomycin or a rise in the extracellular Ca^{2+} concentration. Ionomycin was added to RINm5F cells incubated in Ca^{2+}-containing Hepes buffer supplemented with 0.2 % DMSO (A) or 10 nM TPA (B). In C and D the effects of the indicated concentrations of DMSO and TPA are demonstrated. In the latter cases 2 mM Ca^{2+} was added to a Ca^{2+}-deficient medium in the presence of 25 mM K^+ and 50 µM D-600.

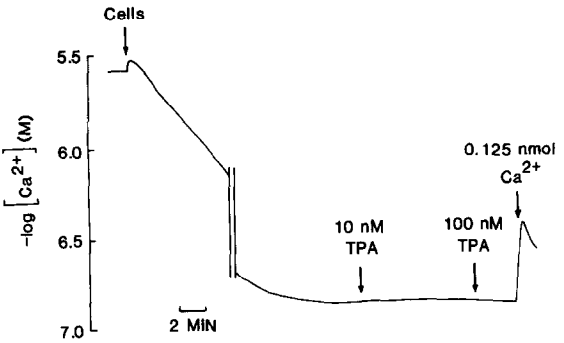

Fig. 3. Effects of successive additions of 10 and 100 nM TPA and 0.125 nmol Ca^{2+} on the ambient Ca^{2+} concentration maintained by permeabilized RINm5F cells. The addition of cells as well as TPA and Ca^{2+} is indicated by the arrows.

these studies also revealed that TPA increased $^{45}Ca^{2+}$ efflux under a variety of experimental conditions such as in the absence of extracellular Ca^{2+} (10) and in the presence of tolbutamide (20). Direct measurements of $[Ca^{2+}]_i$ in RINm5F cells, using the Ca^{2+} indicator quin-2, have suggested that the lowering effect of PKC on $[Ca^{2+}]_i$ is due to a block of the voltage-activated Ca^{2+} channels (17). This could however not be verified from patch-clamp studies, demonstrating that TPA induced an increase in the inward Ca^{2+} currents (21). Alternatively, the lowering effect of protein kinase C activation on $[Ca^{2+}]_i$ might reflect increased intracellular uptake of Ca^{2+}. However, as can be observed in Figure 3, neither 10 nor 100 nM TPA had any effect on the ambient Ca^{2+} concentration maintained by electropermeabilized RINm5F cells. In this context it should be mentioned that TPA also failed to affect Ca^{2+} release induced by $InsP_3$ under similar conditions (11).

We now present experimental data which strongly suggest that the TPA-induced lowering in $[Ca^{2+}]_i$ in RINm5F cells is accounted for by PKC activated Ca^{2+} efflux to the extracellular space. Non-specific Ca^{2+} entrance into RINm5F cells, incubated in Ca^{2+} containing medium supplemented with either 0.2 % DMSO (Figure 2A) or 10 nM TPA (Figure 2B), was stimulated with the Ca^{2+} ionophore ionomycin. Cells incubated in the presence of TPA displayed a slower increase in as well as a lower final level of $[Ca^{2+}]_i$, compared to control cells. In figures 2C and D, $[Ca^{2+}]_i$ was instead increased by raising the extracellular Ca^{2+} concentration from about 10 μM to 2 mM, in a medium containing D-600 to block the voltage-activated Ca^{2+} channels. It was verified that the Ca^{2+} channels indeed were blocked since a high concentration of K^+, included from the beginning of the experiment, was without effect on $[Ca^{2+}]_i$, the latter remaining at the substimulatory level as evident from both figures 2C and D. Also under these conditions, TPA delayed the increase in $[Ca^{2+}]_i$ and the final level of $[Ca^{2+}]_i$ was lower than in control cells. Hence, these data suggest that

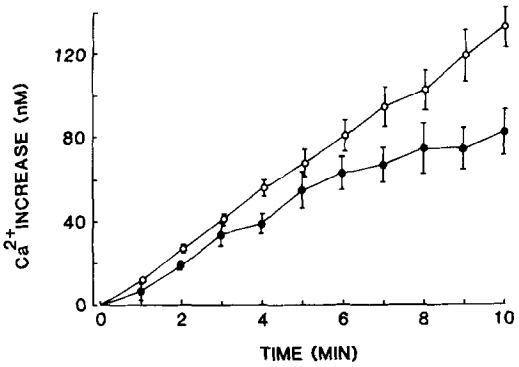

Fig. 4. Effects of DMSO or TPA on Ca^{2+} efflux. RINm5F cells were incubated in a Ca^{2+}-deficient medium and Ca^{2+}-efflux was measured with fura-2 subsequent to stimulation with 0.2 % DMSO (●-●) or 10 nM TPA (O-O) for the indicated period of time. Mean values ± S.E.M. for 3 experiments.

stimulation with phorbol ester promotes a pronounced withdrawal of Ca^{2+} from the cytoplasm. It is technically difficult to directly measure extrusion of Ca^{2+} from intact RINm5F cells subsequent to PKC activation. However, when the ambient Ca^{2+} concentration was measured with fura-2 in a Ca^{2+} deficient medium, we were able to demonstrate that 10 nM TPA indeed stimulated Ca^{2+} efflux to the extracellular space (Figure 4). Although not being directly comparable a previous study, using Arsenazo III and a ten times higher concentration of phorbol ester, has suggested that TPA stimulates Ca^{2+} extrusion from RINm5F cells (22). A plausible candidate responsible for the extrusion of Ca^{2+} is the plasma membrane ATP-dependent Ca^{2+} pump, which has been found to be stimulated by TPA in plasma membrane-enriched fractions from neutrophils (23). This might partly explain why TPA prevented the rise in $[Ca^{2+}]_i$ normally obtained in response to the chemotactic peptide fMet-Leu-Phe in these cells. Hence, the fact that TPA stimulates Ca^{2+} extrusion from RINm5F cells is compatible with the notion that PKC functions as a negative-feedback modulator of stimulated Ca^{2+} influx. Moreover, since DAG is formed in insulin-producing cells in response to a variety of stimuli, including glucose (24), activation of PKC might be part of the complex machinery regulating the insulin secretory process.

ACKNOWLEDGMENTS

This work was supported by grants from the Swedish Medical Research Council (19X-00034), the Bank of Sweden Tercentenary Foundation, the Swedish Diabetes Association, Novo Industry, Swedish Hoechst, Funds of the Karolinska Institute, Farmitalia Carlo Erba, the Nordic Insulin, Clas Groschinskys, Tore Nilsons, Aage Louis-Hansens, Family Ernfors and Magnus Bergvalls Foundations.

REFERENCES

1. Berridge, M.J., and Irvine, R.F. (1984) Nature 312, 315-321.
2. Streb, H., Irvine, R.F., Berridge, M.J., and Schulz, I. (1983) Nature 306, 67-69.
3. Nishizuka, Y. (1984) Nature 308, 693-698.
4. Tanigawa, K., Kuzuya, H., Imura, H., Taniguchi, H., Baba, S., Takai, Y., and Nishizuka, Y. (1982) FEBS Lett. 138, 183-186.
5. Thams, P., Capito, K., and Hedeskov, C.J. (1984) Biochem. J. 221, 247-253.
6. Arkhammar, P., Nilsson, T., Welsh, M., Welsh, N., and Berggren, P.-O. (1989) Biochem. J. in press.
7. Lord, J.M., and Ashcroft, S.J.H. (1984) Biochem. J. 219, 547-551.
8. Hutton, J.C., Peshavaria, M., and Brocklehurst, K.W. (1984) Biochem. J. 224, 483-490.
9. Virji, M.A.G., Steffes, M.W., and Estensen, R.D. (1978) Endocrinology 102, 706-711.
10. Malaisse, W.J., Sener, A., Herchuelz, A., Carpinelli, A.R., Poloczek, P., Winand, J., and Castagna, M. (1980) Cancer Research 40, 3827-3831.
11. Arkhammar, P., Nilsson, T., and Berggren, P.-O. (1986) Biochim. Biophys. Acta 887, 236-241.
12. Hellman, B. (1975) Endocrinology 97, 392-398.
13. Hoftiezer, V., Berggren, P.-O., and Hellman, B. (1985) Cancer Lett. 27, 7-14.
14. Arkhammar, P., Nilsson, T., Rorsman, P., and Berggren, P.-O. (1987) J. Biol. Chem. 262, 5448-5454.
15. Grynkiewicz, G., Poenie, M., and Tsien, R.Y. (1985) J. Biol. Chem. 260, 3440-3450.
16. Tsien, R.Y., and Rink, T.J. (1981) J. Neurosci. Methods 4, 73-86.
17. Di Virgilio, F., Pozzan, T., Wollheim, C.B., Vicentini, L.M., and Meldolesi, J. (1986) J. Biol. Chem. 261, 32-35.
18. Yada, T., Russo, L.L., and Sharp, G.W.G. (1989) J. Biol. Chem. 264, 2455-2462.
19. Wollheim, C.B., Dunne, M.J., Peter-Riesch, B., Bruzzone, R., Pozzan, T., and Petersen, O.H. (1988) EMBO J. 7, 2443-2450.
20. Henquin, J.-C., Bozem, M., Schmeer, W., and Nenquin, M. (1987) Biochem. J. 246, 393-399.
21. Rorsman, P., Arkhammar, P., and Berggren, P.-O. (1986) Am. J. Physiol. 251, C912-C919.
22. Gylfe, E. (1989) Acta Physiol. Scand. 135, 107-111.
23. Lagast, H., Pozzan, T., Waldvogel, F.A., and Lew, P.D. (1984) J. Clin. Invest. 73, 878-883.
24. Peter-Riesch, B., Fathi, M., Schlegel, W., and Wollheim, C.B. (1988) J. Clin. Invest. 81, 1154-1161.