Vol. 131, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1985 Pages 469-476

VASOPRESSIN INDUCED PRODUCTION OF INOSITOL TRISPHOSPHATE AND CALCIUM EFFLUX IN A SMOOTH MUSCLE CELL LINE

Valerie M. Doyle and Urs T. Rüegg

Preclinical Research, Sandoz Ltd., Basel, Switzerland

Received July 3, 1985

Phosphatidylinositol metabolism and $^{45}Ca^{2+}$ efflux were examined in a vascular smooth muscle cell line (A_7r_5). [Arg^8]Vasopressin stimulated the rapid formation (measurable at 1 sec) of inositol phosphates in a concentration-dependent manner. The time course for formation of inositol phosphates was similar to that for $^{45}Ca^{2+}$ efflux from preloaded cells. The efflux of $^{45}Ca^{2+}$ in response to [Arg^8]vasopressin could be inhibited by a vasopressin antagonist. This supports the hypothesis that inositol 1,4,5-trisphosphate plays a role in vasopressin stimulated calcium mobilisation from an intracellular source in cultured vascular smooth muscle cells.

[Arg^8]Vasopressin (AVP) is a peptide originating from the hypothalamus. The two main sites af action are the kidney where it has an antidiuretic effect and smooth muscle where is has a vasoconstrictor effect. Michell et al. (1) have proposed that the vasoconstrictor effect is mediated by V_1 receptors which cause an increase in intracellular calcium $[Ca^{2+}]_i$ while the antidiuretic effect is mediated by V_2 receptors which stimulate cyclic AMP. AVP at concentrations as low as 10^{-12}M can cause contraction of vascular smooth muscle in vitro (2). In liver, stimulation of the V_1 receptor promotes the rapid breakdown of inositol lipids (3-5) resulting in the formation of inositol 1,4,5-trisphosphate ($InsP_3$) which is responsible for the discharge of Ca^{2+} from an intracellular store, probably the endoplasmic reticulum (6-8). AVP has also been shown to increase $InsP_3$ levels and to mobilise $[Ca^{2+}]_i$ in hepatocytes (9), and in platelets (10).

0006-291X/85 $1.50

In this study a smooth muscle cell line (A_7r_5) originating from rat thoracic aorta has been used to examine the effect of AVP on phosphatidylinositol turnover and on $^{45}Ca^{2+}$ efflux. When originally isolated, the cell line was described as having many of the properties characteristic of smooth muscle cells (11). Recent reports have demonstrated the presence of a $Na^+/K^+/Cl^-$ cotransport system (12) as well as a potential sensitive calcium channel (13). The present study provides evidence that this cell line can be used as a model for investigation of events linking receptor activation and intracellular calcium release.

MATERIALS AND METHODS

Materials. [^{3}H]Inositol and $^{45}Ca^{2+}$ were obtained from Amersham International, England. [Arg^8]vasopressin, and the vasopressin antagonist were obtained from Bachem Ltd, Bubendorf, Switzerland. All other chemicals used were of the highest purity grade available.

Cell culture. The rat aortic smooth muscle cell line A_7r_5 was obtained from the American Type Culture Collection, Bethesda, Md. The cells were cultured in a 95% O_2/5% CO_2 incubator in Dulbecco's modified Eagles medium containing 7.5% fetal calf serum. At confluence the cells were trypsinised and subcultured at a density of 7000 cells/cm^2 in dishes of 16 mm diameter. Experiments were carried out on confluent monolayers of cells between the 7 and 14th day after plating.

Phosphatidyl inositol metabolism. Cells were incubated with [^{3}H]inositol (6 µCi/well) for 60 h, long enough for the phospholipids to be labeled to constant specific activity. Following a 3 x 0.5 ml wash with Hepes buffered physiological salt solution (HBPSS in mM: NaCl 145, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1.2, Hepes 5, glucose 10; pH 7.4 at 37°C), the cells were incubated for 10 min with HBPSS containing 50 mM LiCl. Stimulation with agonists was carried out at 37°C for 10 min. The reaction was terminated by the addition of a 95°C solution of 0.1% sodium dodecyl sulphate (SDS) / 10 mM EDTA which was maintained at 95°C for 10 min. After ultrasonication for 2 min, the lysates were centrifuged and the supernatants were analysed for the presence of [^{3}H]inositol phosphates by stepwise elution from anion exchange columns and scintillation counting (14).

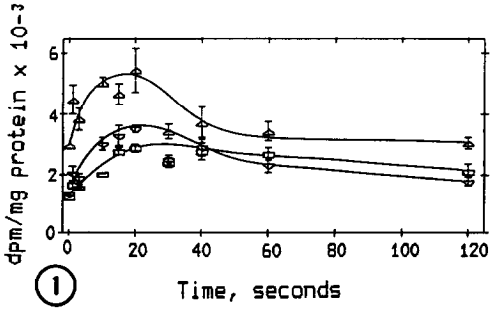
$^{45}Ca^{2+}$ loading and efflux. Cells were washed twice with HBPSS and incubated for 30 min in an isotonic HBPSS containing 55 mM KCl and $^{45}Ca^{2+}$ at 37°C. The cells were rapidly washed four times with ice cold HBPSS. Efflux was measured in HBPSS containing 0.1% BSA (37°C) for 10 min after which peptides were added and efflux was continued for an additional 10 minutes. The incubation was

terminated by washing the cells in ice cold HBPSS followed by the addition of 0.1% SDS. An aliquot was counted to determine the $^{45}Ca^{2+}$ content remaining in the cells. The remainder was used for protein estimation using BSA as standard (15).

RESULTS

AVP (10^{-7}M) increased the production of $InsP_3$, $InsP_2$ and $InsP_1$ (Fig. 1). Increases in $InsP_3$ were observed after 1 sec, reached a plateau at 20 sec and then decreased to basal level at 2 min. $InsP_2$ and in particular $InsP_1$ accumulated over longer periods of time. Such a transient rise in the production of $InsP_3$ does not permit accurate measurements, since the rate of association of AVP with the receptor is too low (16). However, in the presence of LiCl (50 mM, isotonic) a linear accumulation of all three inositol phosphates was observed in the presence of AVP. Using a stimulation period of 10 min, concentration response curves for the accumulation of the inositol phosphates were obtained (Fig. 2). The threshold concentration of AVP that generated a detectable increase in inositol phosphate formation was between 1 and 3 x 10^{-10}M. A slight difference in EC_{50} values for production of $InsP_3$, $InsP_2$ and $InsP_1$ was observed; the values were 3 x 10^{-9} M, 3 x 10^{-9}M and 10^{-9}M respectively for the three products. AVP stimulated formation of inositol polyphosphates was inhibited by the addition of an AVP antagonist (Table 1). Measurement in the presence of EGTA (5 mM in HBPSS) did not affect the production of $InsP_3$, $InsP_2$ or $InsP_1$ by AVP.

The kinetics of $^{45}Ca^{2+}$ efflux from preloaded cells in physiological solution alone or in the presence of AVP (10^{-7}M) are shown in Fig. 3. In the absence of a stimulant at least two phases can be distinguished. The initial phase is very rapid, causing an efflux of 70% of cellular $^{45}Ca^{2+}$ at a rate of



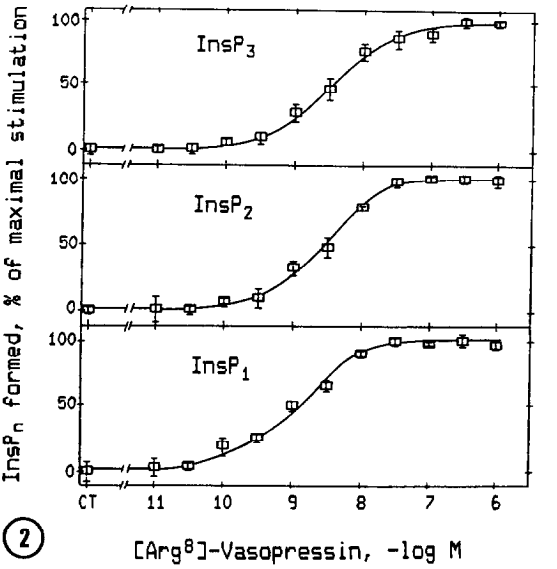


Figure 1: Time course of the formation of inositol phosphates: $InsP_3$ (Δ), $InsP_2$ (∇), and $InsP_1$ (□). Cells prelabelled for 60 hours with [^{3}H]inositol were washed and stimulated with 0.1 μM AVP. After the times indicated, boiling 0.1% SDS/10 mM EDTA was added and the cellular lysate analysed as described as in Methods.

Figure 2: Concentration response curves for the formation of the three inositol phosphates. Cells were labelled as in Fig. 1. Before the addition of AVP, cells were preincubated for 10 min in isotonic HBPSS containing 50 mM LiCl. Following a 10 min incubation of the cells with AVP (0.1 μM), the cells were lysed and analysed as in Methods.

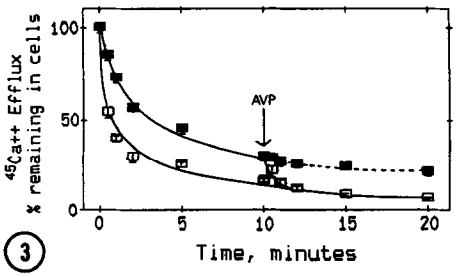
8.1×10^{-3} s^{-1}; the second phase is slower and has a rate of 0.34×10^{-3} s^{-1}. In the presence of AVP (10^{-7}M), the initial phase was accelerated, resulting in an 80% efflux at a rate of 20×10^{-3} s^{-1}, while the slower phase had a rate of 1×10^{-3} s^{-1}. However, the early rapid phase tended to mask the specific efflux mediated by AVP. In order to diminish this effect, efflux was carried out for 10 min in HBPSS, after which AVP (10^{-7}M) was added. This reduced by 70% the $^{45}Ca^{2+}$ remaining in the cells within 5 min (Fig. 3). Preincubation with 50 mM LiCl had no effect on either basal or stimulated $^{45}Ca^{2+}$ efflux. The AVP antagonist [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),Tyr (Me)^2,

Table 1

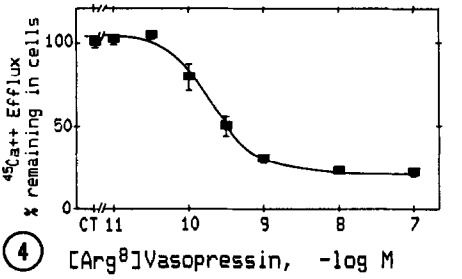
Antagonism of AVP (10^{-7}M) induced inositol phosphate production and AVP (10^{-9}M) induced $^{45}Ca^{2+}$ efflux by a V_1 antagonist (see text). Results are expressed as mean ± sem dpm per culture well; n = 4.

	HBPSS	AVP	AVP+antagonist (10^{-7}M)
[^{3}H]Inositol phosphate production			
$InsP_1$	352 ± 25	3336 ± 404	852 ± 46
$InsP_2$	305 ± 15	2856 ± 309	354 ± 10
$InsP_3$	367 ± 6	841 ± 97	409 ± 11
$^{45}Ca^{2+}$ remaining in the cells			
$^{45}Ca^{2+}$	1512 ± 72	652 ± 14	1478 ± 136

Val^4, D-Arg^8]vasopressin (17), added 10 min prior to AVP, had no effect on efflux in HBPSS but antagonised the AVP mediated efflux (Table 1). Fig. 4 shows the concentration dependent efflux of $^{45}Ca^{2+}$ mediated by AVP, the threshold concentration that resulted



3



4

Figure 3: Time course of calcium efflux. Cells were loaded to equilibrium with $^{45}Ca^{2+}$, washed at 4°C and efflux was studied at 37°C in HPBSS (■) or in the presence of 0.1 μM AVP (□), added at time 0 or 10 min. The buffer was aspirated and the $^{45}Ca^{2+}$ remaining was determined.

Figure 4: Calcium efflux: concentration response relationship. 100% represents the $^{45}Ca^{2+}$ remaining in the cells after 20 min efflux in HPBSS. The addition of AVP (0.1 μM) after 10 min (Fig. 3) reduced cell associated $^{45}Ca^{2+}$ to 25%.

in a detectable efflux was between 0.3 and 1 x 10^{-10}M; the EC_{50} value for efflux was 0.3 x 10^{-9}M.

DISCUSSION

Freshly isolated smooth muscle cells contract in response to stimuli such as AVP (16) or angiotensin II (18) indicating the presence of a fully functional receptor-response cascade. However, after several passages in vitro, these cells loose their ability to contract (19). The reason appears to be a loss of myosin (19); other early steps leading to contraction are however still present. We wish to demonstrate with this report that the A_7r_5 cell line, which is also devoid of a functional contractile apparatus, can be used as a model for the study of the early steps leading to contraction.

AVP at concentrations of 10^{-10} to 10^{-7}M induced the production of inositol phosphates and $^{45}Ca^{2+}$ efflux from these cells. These results are consistent with data previously published in other cell types (8-10) and with the recent reports on cultured arterial muscle cells that are responsive to angiotensin II (20-22) and to AVP (21).

Analysis of basal $^{45}Ca^{2+}$ efflux suggested the presence of at least two kinetic phases (Fig. 3). AVP accelerated the calcium release from both components; it is very likely that at least one component is due to release from the endoplasmic reticulum, which is the main intracellular pool from which calcium is liberated (6-8). Addition of AVP after the decay of the first rapid phase (10 min) resulted in a 70% increase in efflux of cell associated $^{45}Ca^{2+}$. This increased efflux was inhibited by a V_1 antagonist, indicating that the effect is mediated by the V_1 type receptor (1,5). These results are consistent with previous observations

suggesting that interaction of the agonist with its membrane receptor leads to the production of $InsP_3$ which is causing the release of $^{45}Ca^{2+}$ sequestered intracellularly (6-8). The EC_{50} values for AVP mediated $InsP_3$ production and Ca^{2+} efflux, 3 nM and 0.3 nM respectively, suggest that only a small amount of $InsP_3$ is required for maximal calcium mobilisation indicating the presence of a receptor reserve. Another factor which has recently come to light is that the $InsP_3$ measured may not be all Ins 1,4,5-P_3 but may in part be the isomer Ins 1,3,4-P_3 (23) whose function is at present unknown.

In the absence of lithium, $InsP_3$ levels decay within one to two minutes; the decay of $InsP_2$ and $InsP_1$ is slower (Fig. 1). This is in favour of a step-to-step degradation by the respective phosphatases (24). The fact that high concentrations of LiCl (50 mM) also inhibits $InsP_3$ and $InsP_2$ degradation (Fig. 2) points to a possible product inhibition of each of the phosphatases since it is thought that only the inositol 1-phosphate phosphatase is inhibited by lithium ion (24). In liver cells, a similar accumulation of the early products has also been observed (9). We believe that it is for this reason that the response with respect to $InsP_1$ formation is somewhat more sensitive (EC_{50} ca. 1 nM) than for $InsP_2$ and $InsP_3$ (EC_{50} 3 nM; Fig. 2).

In summary, we present evidence that stimulation of the AVP receptor results in increased inositol phosphate metabolism and intracellular calcium release; these early events of the receptor-response cascade can be studied quantitatively in this smooth muscle cell line. Therefore, this cell line appears to be a suitable model for the examination of the initial events involved in the calcium induced contraction process.

ACKNOWLEDGEMENTS

We would like to thank Drs. U. Quast and B. Gähwiler for advice and help and Mr. J. Peter and Mr. J.F. Zuber for expert technical assistance.

REFERENCES

1. Michell, R.H., Kirk, C.J. and Billah, M.M. (1979) Biochem. Soc. Trans. 7, 861-865.
2. Altura, B.M. and Altura, B.T. (1977) Fed. Proc. 36, 1853-1860.
3. Billah, M.M. and Michell, R.H. (1979) Biochem. J. 182, 661-668.
4. Thomas, A.P., Marks, J.S., Coll, K.E. and Williamson, J.R. (1983) J. Biol. Chem. 258, 5716-5725.
5. Creba, J.A., Downes, C.P., Hawkins, P.T., Brewster, G., Michell, R.H. and Kirk, C.J. (1983) Biochem. J. 212, 733-747.
6. Streb, H., Irvine, R.F., Berridge, M.J. and Schultz, I. (1983) Nature, 306, 67-69.
7. Burgess, G.M., Godfrey, P.P., McKinney, J.S., Berridge, M.J., Irvine, R.F. and Putney, J.W. Jr. (1984) Nature 309, 63-66.
8. Suematsu, E., Hirata, M., Hashimoto, T. and Kuriyama, M. (1984) Biochem. Biophys. Res. Commun. 120, 481-485.
9. Thomas, A.P., Alexander, J. and Williamson, J.R. (1984) J. Biol. Chem. 259, 5574-5584.
10. McIntyre, D.E. and Pollock, W.K. (1982) Brit. J. Pharmacol. 77, 466.
11. Kimes, B.W. and Brandt, B.L. (1976) Exp. Cell Res. 98, 349-366.
12. Owen, N.E. (1984) Biochem. Biophys. Res. Commun. 125, 500-508.
13. Rüegg, U.T., Doyle, V.M., Zuber, J.F. and Hof, R.P. (1985) Biochem. Biophys. Res. Commun. (in press).
14. Berridge, M.J., Dawson, R.M.C., Downes, C.P., Heslop, J.P. and Irvine, R.F. (1983) Biochem. J. 212, 473-482.
15. Lowry, O.H., Rosebrough, J.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
16. Penit, J., Faure, M. and Jard, S. (1983) Amer. J. Physiol. 244, E72-82.
17. Manning, M., Lammek, B. and Kolodziejczyk, A.M. (1981) J. Med. Chem. 24, 701-706.
18. Gunther, S., Alexander, R.W., Atkinson, W.J. and Gimbrone, M.A. (1982) J. Cell Biol. 92, 289-298.
19. Chamley-Campbell, J., Campbell, G.R. and Rose, R. (1979) Physiol. Rev. 59, 1-61.
20. Smith, J.B., Smith, L., Brown, E.R., Barnes, D. Sabir, M.A., Davis, J.S. and Farese, R.V. (1984) Proc. Natl. Acad. Sci. USA 81, 7812-7816.
21. Nabika, T., Velletri, P.A., Lovenberg, W. and Beaven, M.A. (1985) J. Biol. Chem. 260, 4661-4670.
22. Alexander, R.W., Brock, T.A., Gimbrone, M.A. and Rittenhouse, S.E. (1985) Hypertension 7, 447-451.
23. Irvine, R.F., Letcher, A.J., Lander, D.J. and Downes, C.P. (1984) Biochem. J. 223, 237-243.
24. Berridge, M.J. (1984) Biochem. J. 220, 345-360.