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EFFECTS OF HEPARIN ON INOSITOL 1,4,5- TRISPHOSPHATE AND GUANOSINE 5'-O- (3-THIO TRIPHOSPHATE) INDUCED CALCIUM RELEASE IN CULTURED SMOOTH MUSCLE CELLS FROM RABBIT TRACHEA

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SUMMARY: The effects of heparin on intracellular calcium release in monolayers of permeabilised cultured rabbit smooth muscle cells were determined using ^{45}Ca effluxes. Low molecular weight heparin inhibited inositol 1,4,5- trisphosphate ($InsP_3$) induced Ca^{2+} release (IC_{50} = 0.8μg/ml), but not guanosine 5'-O- (3-thio triphosphate) (GTPγS) stimulated Ca^{2+} release. Only a small inhibition was noted with high molecular weight heparin and de-N-sulphated heparin, although chondroitin sulphate A potently inhibited the $InsP_3$ response. These results indicate the competitive and specific nature of the heparin effect and give information about the structure of the $InsP_3$ binding site.

Inositol 1,4,5-trisphosphate ($InsP_3$) is an important secondary messenger releasing intracellularly stored calcium (1). The nature of the calcium release mechanism is still not understood, but may involve GTP-dependent proteins. Guanosine 5'- triphosphate (GTP) or nonhydrolysable GTP analogues such as Guanosine 5'-O-(3-thiotriphosphate) (GTPγS) have also been found to release intracellular calcium in a number of cells (2,3,4), but now it seems that $InsP_3$ induced calcium release and GTP stimulated calcium release act by different mechanisms (5).

Heparin has diverse effects in a variety of cell types, it can inhibit the effect of adrenaline on adenylate cyclase in membranes of human platelets (6) and can block $InsP_3$ binding to cerebellar membrane fractions (7). In permeabilised rat liver cells heparin inhibits $InsP_3$ induced calcium release (8), and in rat liver microsomes it is found that heparin, although strongly inhibiting $InsP_3$ promoted Ca^{2+} release has no effect on the release promoted by GTP (4).

In vascular smooth muscle cells, heparin inhibits the $InsP_3$ - dependent but not the -independent calcium release induced by guanine nucleotide (9). Permeabilised cells of the smooth muscle cell line DDT_1MF-2 show that heparin potently and competitively

ABBREVIATIONS: $InsP_3$, inositol 1,4,5-trisphosphate; GTP, guanosine 5'-triphosphate; GTPγS, guanosine 5'-O- (3-thio triphosphate); EGTA, ethylene glycol-bis (β-aminoethylether)N,N,N,N-tetraacetic acid.

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antagonises $InsP_3$ induced calcium release but has no effect on the GTP-activated Ca^{2+} translocation. Studies using De-N-sulphated heparin in these cells show no effect on $InsP_3$ induced calcium release indicating the structural specificity of the $InsP_3$ binding site (10).

The aim of this study was to investige the effect of heparin on the release of intracellular Ca^{2+} induced release by $InsP_3$ in permeabilsed airway smooth muscle cells, and on the Ca^{2+} release induced by GTPγS. Using heparin analogues we have studied the structural specificity for the inhibition of response.

MATERIALS AND METHODS

Cell culture: Smooth muscle cells (passages 5-10) isolated from rabbit trachea were cultured using an identical technique to that described in detail for human airway smooth muscle (11). Cells were subcultured onto 35mm dishes and grown to confluency (0.2mg cell protein /dish) in Dulbecco's Modified Eagle's medium supplemented by 10% foetal calf serum, penicillin 100U/ml and streptomycin 100μg/ml. (37°C, 95% air/ 5% CO_2, water saturated).

Ca^{2+} efflux from skinned cells: All experiments were performed at room temperature (22°C) and ethylene glycol-bis(ß-aminoethylether)N,N,N,N-tetraacetic acid (EGTA) solutions also contained KCl 130mM, $MgCl_2$ 5mM, Tris-maleate buffer 20mM, pH 6.8.

The tissue culture medium was removed from 35mm petri-dishes containing confluent monolayers of cells. The cells were then washed repeatedly, first with physiological salt solution (PSS) and then in 4mM EGTA for 2 minutes. They were then incubated for 15 minutes in an otherwise identical solution but containing digitonin (40μM) in order to permeabilise the plasmalemma. Any cellular calcium would be removed by the 4mM EGTA.

The digitonin was removed by washing in a 0.1mM EGTA solution for a further 2 minutes. Cells were then incubated for 30 minutes in ^{45}Ca-labelled Ca^{2+} solution (10μCi ^{45}Ca/ml) with a free calcium concentration of 1μM. Na_2ATP (3.15mM) was added to the solution in order to facilitate active transport of calcium into the sarcoplasmic reticulum.

Calcium uptake by the permeabilsed cells was stopped by removal of the labelling solution. ^{45}Ca was effluxed from the cells into 0.1mM EGTA . This solution was changed every minute for 21 minutes and each aliquot collected in a vial. In test dishes heparin (0.1-10μg/ml) was added to the effluxing solution from 5-15 minutes inclusive and $InsP_3$ (0.5-20μM) was added for 1 minute at 15 mins. Control dishes had either no heparin added and were stimulated by maximally effective $InsP_3$(10μM) or had maximally effective heparin added (10μg/ml) but no $InsP_3$. Blank controls were incubated with normal efflux solution only.

In GTPγS experiments, the procedure was identical except that the efflux solution was changed every minute for 10 minutes, and then every 5 minutes for 55 minutes (GTPγS has a longer response time than $InsP_3$). Heparin was added from 25-45 minutes inclusive, GTPγS was added at 35 minutes for 10 minutes, and InsP3 + GTPγS added at 45 minutes for 5 minutes.

Cells were harvested from the dishes at the end of the experiment using PSS containing collagenase (1mg/ml) and trypsin (0.1mg/ml). The radioactivity of the cells, of each vial and the labelling solution was estimated using liquid scintillation counting. The amount of protein in the cells was measured by the method of Lowry et al. (12)

from dishes cultured at the same time as the test dishes. The Ca^{2+} content was expressed as nmol Ca/mg protein, and Ca^{2+} loss as nmol Ca/mg protein/minute. By calculating the decrease in calcium content on addition of $InsP_3$ or GTPγS, and correcting for control efflux at this time point, values for Ca^{2+} release on addition of stimulant could be calculated.

Materials: GTPγS, $InsP_3$, heparin, low molecular weight heparin (MW 4,000-6000), De-N-sulphated heparin, and chondroitin sulphate A were purchased from Sigma. Tissue culture reagents were purchased from Gibco. All other reagents were of the highest grade commercially available.

RESULTS

The rate of Ca^{2+} loss from the monolayers fell progressively during the efflux procedure. The initial high rate of efflux was assumed to be loss of loosely bound extracellular followed by loss of cytoplasmic Ca^{2+}. By 10 min, the Ca^{2+} loss occured predominantly from the intracellular store which was slowest to efflux and the fall in Ca content became monoexponential (13).

Maximally effective $InsP_3$(10μM), released 1.3nmol ± 0.23 Ca^{2+}/mg protein (n=7). Exposure to low molecular weight heparin (10μg/ml) reduced the $InsP_3$ response to 0.015nmol ± 0.02 Ca^{2+}/mg protein (n=3) (Figure 1). Heparin on its own showed no stimulation or release of calcium.

Maximally effective GTPγS (100μM) released 0.51nmol Ca^{2+} ± 0.23/mg protein (n=5). Low molecular weight heparin had no effect on the GTPγS response (Figure 2), indicating the specificity of the heparin response to the $InsP_3$ binding site.

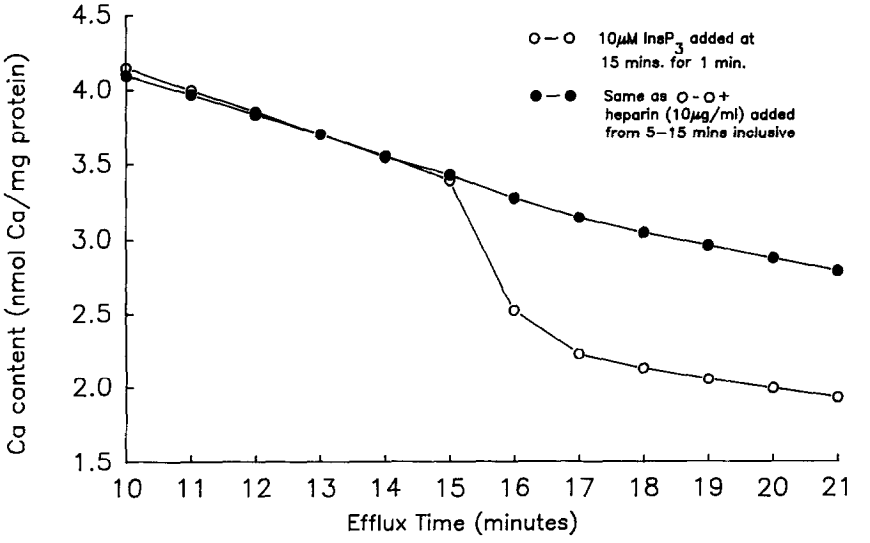

Figure 1. Reduction in calcium content of monolayers of permeabilised cells induced by 10μM $InsP_3$ in the absence and presence of 10μg/ml low molecular weight heparin. Heparin completely inhibited $InsP_3$ induced Ca release. The plot shows the results of a single experiment.

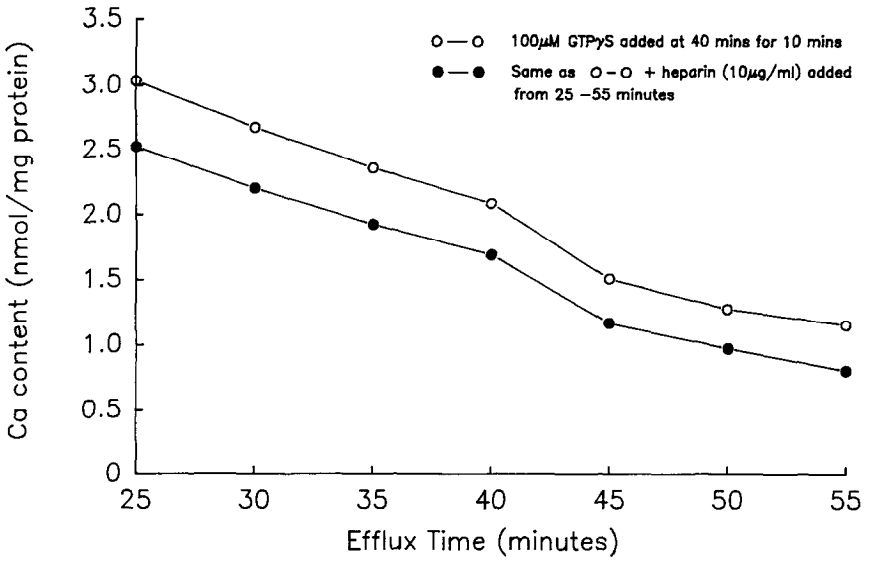

Figure 2. Reduction in calcium content of monolayers of permeabilised cells induced by 100μM GTPγS in the absence and presence of 10μg/ml low molecular weight heparin. The overall calcium content in the heparin experiment is lower due to different loading of ^{45}Ca between dishes, the rate of efflux however remains the same. Heparin had no effect on GTPγS induced calcium release.

The sensitivity of the heparin effect was studied by varying the concentrations of heparin from 0-10 μg/ml and noting the inhibition of maximally effective $InsP_3$ (Figure 3). Half maximal inhibition of Ca^{2+} release stimulated by 10μM $InsP_3$ occured with heparin at a concentration of 0.8μg/ml or approximately 160nM (assuming an average MW of 5000).

The dose response to $InsP_3$ (0.5-20μM) was carried out with and without heparin at 0.2μg/ml. A Lineweaver-Burk plot (Figure 4) illustrates that the intercept on the x axis corresponding to -1/Km apparent changes, but the intercept on the y axis

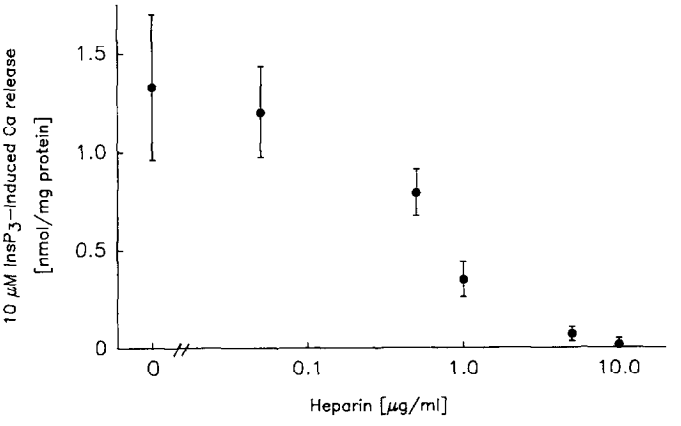

Figure 3. Dose dependent inhibition of 10μM InsP3 induced calcium release by low molecular weight heparin. Results are expressed as means of 3 experiments ± S.D.

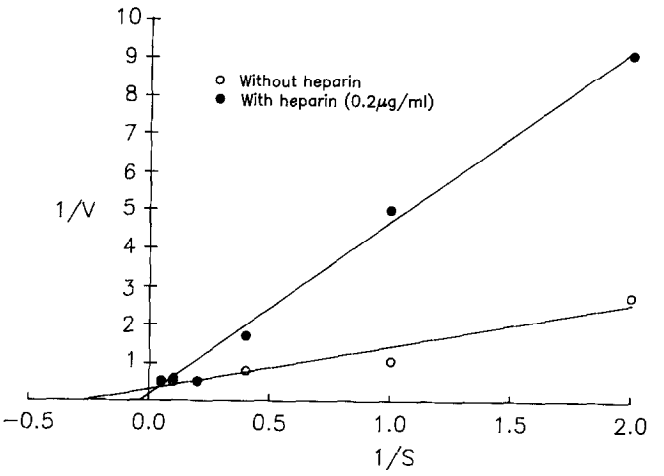

Figure 4. Lineweaver - Burk plot of $InsP_3$ dose response curves performed in the absence and presence of 0.2μg/ml low molecular weight heparin. 1/S represents $1/InsP_3$ conc.(μM), 1/V represents 1/calcium release (nmol Ca/mg protein). This is an example of one experiment.

corresponding to 1/Vmax remains the same, indicating the competitive nature of the heparin effect on the $InsP_3$ response. Using cumulative data, Km with and without heparin was found to be 8.17 ± 2.08 nM and 2.14 ± 0.37 nM respectively ($p<0.05$, n=3). Vmax with and without heparin was 2.04 ± 0.36 nmol Ca^{2+} and 1.77 ± 0.13 nmol Ca^{2+} (n=3), and there was no significant difference. From this data and assuming a heparin concentration of 40nM, Ki was calculated as 13.3nM.

These results suggest that heparin competes for a common binding site with $InsP_3$. This was further investigated by studying the effects of heparin analogues on $InsP_3$ response. It was noted that De-N-sulphated heparin and high molecular weight heparin showed only a very small inhibition of response. Chondroitin sulphate A however, showed a very potent inhibition of $InsP_3$ response (Table 1) These results give an indication of the residues involved at the $InsP_3$ active site.

TABLE 1. Inhibition of $InsP_3$ induced Ca release by structural analogues of heparin

ANALOGUE (conc.10μg/ml)	% INHIBITION OF MAXIMUM $INSP_3$ RESPONSE
HEPARIN	12.0 ± 4.1
LOW MW HEPARIN	96.7 ± 4.1
CHONDROITIN SULPHATE A	95.4 ± 4.9
DE-N-SULPHATED HEPARIN	15.5 ± 10.8

Results are expressed as % inhibition of the calcium release induced by maximally effective $InsP_3$ (10μM), and represent the mean ± S.D. of three experiments.

DISCUSSION

We have demonstrated that heparin can potently block intracellular Ca^{2+} release induced by $InsP_3$ but has no effect on that induced by GTPγS in permeabilsed airway smooth muscle cells. The sensitivity of the heparin effect is high, half maximal inhibition of $InsP_3$ induced Ca release occured with low molecular weight heparin at a concentration of 0.8 μg/ml which is comparable to the 0.6 μg/ml, obtained by Ghosh et al, (10) using permeabilised cells from the smooth muscle line DDT_1MF-2. The low Ki value obtained in our experiments (13.3nM) gives an indication of the high affinity that heparin has for the $InsP_3$ receptor.

The specificity of the inhibition using different heparin analogues gives interesting information about the structure of the $InsP_3$ binding site. Chondroitin Sulphate A was found to be very effective at blocking $InsP_3$ induced calcium release in contrast to results obtained by Hill et al, (8) who showed no inhibition of Ca^{2+} mobilisation in permeabilsed rat liver cells with chondroitin sulphate. Since this group did not specify the particular isomer used this could be a major contribution to the differences noted. Nilsson et al (14), however have shown that in permeabilsed pancreatic ß cells, heparin inhibits $InsP_3$ induced calcium release but this effect is not seen with chondroitin sulphate A or C. This may result from the differing duration of incubation with heparin compared to our experiments. We found that cells had to be preincubated for a minimum of 10 minutes for maximal inhibition to occur, no inhibition occuring before 5 mins, (results not shown).

As expected De-N-sulphated heparin showed no inhibition of response (10), suggesting that binding to the $InsP_3$ binding site depends on the relative positions of the different sulphated residues and the N residue on the hexosamine unit. High molecular weight heparin showed only a very small inhibition of the $InsP_3$ response. This may be expected since in order for the molecule to interact with the $InsP_3$ binding site, it may have to be "folded" with certain domains available which may be hindered by many more residues being present. It is interesting however that chondroitin sulphate A which has a high molecular weight of 50,000 caused a potent inhibition of response. This may be due to different "folding" within the molecule making the $InsP_3$ binding domains more readily available.

We conclude that heparin can be a useful tool in investigating $InsP_3$ and GTP interactions and identifying specific $InsP_3$ binding sites. Further work involving heparin analogues may provide more structural information about these sites in airway smooth muscle cells

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REFERENCES

1. Berridge, M.J. and Irvine, R.F. (1984) Nature 312:315-321.
2. Gill, D.L., Ueda, T., Cheuh, S.H. and Noel, M.W. (1986) Nature 320:461-464.
3. Somylo, A.P., Walker, J.W., Goldman, Y.E., Trentham, D.R., Kobayashi, S., Kitazawa, T. and Somylo, A.V. (1988) Phil. Trans. R. Soc. Lond. B 320:399-414.
4. Cullen, P.J., Comerford, J.G. and Dawson, A.P. (1988) FEBS Lett. 228:57-59.
5. Cheuh, S-H., Mullaney, J.M., Ghosh, T.K., Zachary, A.L. and Gill, D.L. (1987) J. Biol. Chem. 262:13857-13864.
6. Willuweit, B. and Aktories, K. (1988) Biochem. J. 249:857-863.
7. Worley, P.F., Baraban, J.M., Supattapone, S., Wilson, V.S. and Snyder, S.H. (1987) J. Biol. Chem. 262:12132-12136.
8. Hill, T.D., Berggren, P-O. and Boynton, A.L. (1987) Biochem. Biophys. Res. Commun. 149:897-901.
9. Kobayashi, S., Somlyo, A.V. and Somlyo, A.P. (1988) Biochem. Biophys. Res. Commun. 153:625-631.
10. Ghosh, T.K., Eis, P.S., Mullaney, J.M., Ebert, C.L. and Gill, D.L. (1988) J. Biol. Chem. 263:11075-11079.
11. Twort, C. and Van Breemen, C. (1988) Tissue and Cell 20:339-344
12. Lowry, O.H., Rosebrough, N.J., Farr, A.C. and Randall, R.J. (1951) J. Biol. Chem. 193:265-275.
13. Yamamoto, H. and Van Breemen, C. (1986) J. Gen. Physiol. 87:369-389
14. Nilsson, T., Zwiller, J., Boynton, L.B. and Berggren, P-O. (1988) FEBS Lett. 229:211-214.