Biochemical Pharmacology, Vol. 31, No. 1, pp. 117–119, 1982.
Printed in Great Britain.

0006-2952/82/010117-03 $03.00/0

In vitro desensitization of human lymphocytes by epinephrine

(*Received* 24 *February* 1981; *accepted* 24 *June* 1981)

Exposure of target tissues to β-adrenergic catecholamines causes both an increase in cAMP synthesis and a time-dependent loss of sensitivity to further stimulation by these same hormones. In man, this agonist-specific desensitization is a target tissue property *in vivo* as well as *in vitro* [1–3], but the mechanism by which it occurs is poorly understood. β-Adrenergic catecholamine desensitization was initially believed to be the consequence of agonist-induced loss of functional cell surface β-adrenergic receptors [4, 5], but more recent evidence of substantive decreases in sensitivity with little or no change in receptor number indicates a more complex mechanism or mechanisms distal to the interaction between hormone and receptor [3, 6, 7].

In general, the rate at which desensitization occurs appears to be hormone and/or cell specific with a time course of days or hours in some cases [8, 9] and minutes in others [2, 3]. Presumably, desensitization protects hormone-sensitive tissues from "over-stimulation", so that rate of progression might be expected to proceed as a function of the seriousness of the consequences of over-stimulation. Using an accessible human target tissue, we found that desensitization of lymphocytes isolated from human subjects undergoing isoproterenol infusion proceeded with rapid time course [3]. Desensitization *in situ* proceeded without detectable changes in β-adrenergic receptor concentration, and similar losses of sensitivity could be induced in the lymphocytes *in vitro*. These results suggested that human tissues have a mechanism for rapidly reducing their sensitivity on exposure to β-adrenergic catecholamines, and we sought to determine how acute this response might be.

Methods

Blood, collected in heparinized tubes, was centrifuged at 1000 *g* and lymphocytes were prepared from the leukocyte layer by centrifugation on gradients of Ficol–Hypaque as described previously [3]. Cells were resuspended in Krebs–Ringer bicarbonate buffer equilibrated with 95% O_2–5% CO_2, and the density was determined by hemocytometer count and verified by DNA analysis [10] using 6.4 pg as the DNA content of the human diploid nucleus. In the standard sensitivity assay, freshly isolated lymphocytes were incubated in gassed Krebs–Ringer bicarbonate at a density of 10 million cells per ml at 37° in the presence of 100 μM (−)-isoproterenol and 5 mM theophylline or theophylline alone (basal). After 7 min, cAMP was extracted by the addition of 9 vol. propanol for quantification by the method in Ref. 3. Under these conditions, isoproterenol increased cAMP content by about 5 pmoles per 1 million cells, a 2.5-fold increase over basal levels.

Lymphocyte adenylate cyclase activity, determined as enzyme-specific conversion of [α-^{32}P]ATP to [^{32}P]cAMP and subsequent purification of the radioactive product by Dowex and alumina ion exchange chromatography, was quantified using whole cells lysed by the hypotonic assay buffer as described in Ref. 11. Enzyme activation by pretreatment with guanyl-5′-yl imidodiphosphate (GMP.P(NH)P) was performed by incubating 5 million cells in the same hypotonic buffer consisting of 1 ml of 0.05 M HEPES* buffer (Na^+ salt), (pH 7.6), 0.001 M EGTA, 10% dimethylsulfoxide, and the desired additions at 4° for 10 min. The suspension was centrifuged at 4° for 20 min at 20,000 *g* and the supernatant fraction was discarded. The particulate fraction was resuspended in the same buffer with no additions at 4° and the washing step was repeated one time. The washed pellet was resuspended for enzyme assay as described above. [^{3}H]Epinephrine binding was performed in a manner identical to activation by pretreatment with epinephrine and GMP.P(NH)P at 4°. Five million hypotonically lysed cells were incubated with 0.1 to 1000 nM radioligand (40 Ci/mmole; New England Nuclear Corp., Boston, MA) at 4° in 1 ml of the buffer described above for periods of between 10 and 30 min. The cells were then centrifugally washed as described and dissolved in 1% sodium dodecylsulfate for scintillation counting. Duplicate aliquots were incubated at each radioligand concentration.

Results

When epinephrine was added to human lymphocytes *in vitro*, there was a large and immediate decrease in the agonist concentration of the medium and within 3 min nearly 85 per cent of the hormone was gone (Fig. 1). Seventy per cent of the remaining epinephrine was

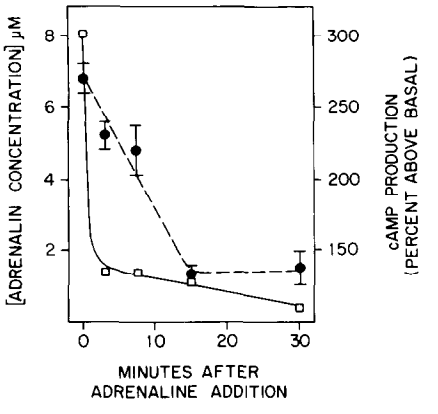

Fig. 1. Epinephrine degradation and agonist-dependent desensitization *in vitro*. Lymphocytes were incubated at 37° in Krebs–Ringer bicarbonate and epinephrine was added at the zero time point. Aliquots of the incubation mixture were withdrawn at the indicated times for determination of the concentration of the catecholamine as the [^{3}H]-*O*-methyl product formed by catechol-*O*-methyltransferase by the method of Peuler and Johnson [12] as described in Ref. 11. Additional aliquots were withdrawn and centrifuged at 1000 *g* for 10 min to concentrate the cells which were centrifugally washed free of the agonist by being resuspended twice in epinephrine-free buffer. Cells from each time interval were then incubated in the presence or absence of 100 μM isoproterenol for determination of their β-adrenergic catecholamine sensitivity, as described in Methods. The results, the mean of duplicate determinations of epinephrine concentration (□) and the mean ± S.D. of three separate determinations of agonist-dependent cAMP production (●), are representative of those obtained with two different preparations.

* Abbreviations: HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; and EGTA, ethyleneglycol bis (amino-ethylether) tetra-acetate.

degraded during the following 27 min. In comparison to the rapid rate of hormone degradation, lymphocyte desensitization proceeded more slowly, with a half-time of about 9 min, a rate slightly slower but not dissimilar to that achieved using the specific β-adrenergic catecholamine agonist isoproterenol (6 min, Ref. 3). Progressive desensitization, in spite of the dramatic decrease in hormone concentration, suggested that the period during which epinephrine interacted with its receptor to initiate desensitization was brief. Since even low concentrations of agonist are desensitizing [3], we adopted procedures to more effectively remove epinephrine from lymphocytes after brief periods of exposure.

Human lymphocytes could be kept at 4° for several hours without impairing their ability to increase cAMP production in response to isoproterenol when warmed again to 37° (not shown). Consequently, we incubated cells for periods up to 20 min in the presence of epinephrine at 4°, followed by dilution with cold agonist-free buffer and rapid centrifugal washing to remove unbound hormone. Following a 30-min incubation at 37°, the cells were tested for their ability to respond to isoproterenol at 37°. Hormone pretreatment at the low temperature did not result in elevated basal cAMP levels in the subsequently washed cells, and exposure for as little as 45 sec was adequate to activate the mechanism which led to a complete or nearly complete loss of sensitivity if the cells were subsequently incubated at 37° for 30 min (Fig. 2). Desensitization by epinephrine pretreatment required subsequent incubation at the elevated temperature and, if it occurred at the low temperature, it proceeded too slowly to be reliably detected.

These results indicated that a functional hormone–receptor complex could be formed at 4°. We tested the ability of agonists to activate the enzyme at that low temperature by pretreating cells at 4° with GMP.P(NH)P in the absence or presence of epinephrine or isoproterenol (Fig. 3). GMP.P(NH)P, a poorly hydrolyzed GTP analog that satisfies the guanyl nucleotide requirements for coupling hormone receptors and the adenylate cyclase catalytic subunit in these human cells [11], also activated the enzyme in an irreversible manner. Consequently, cells pretreated with GMP.P(NH)P at 4° in the hypotonic buffer, and then washed centrifugally in guanyl nucleotide-free buffer, had persistently elevated activity in the absence of additions to the subsequent enzyme assay (Fig. 3). Pretreatment with GMP.P(NH)P at 4° with either isoproterenol or epinephrine led to a significant ($P < 0.01$) further increase in activity of the subsequently washed cells (Fig. 3).

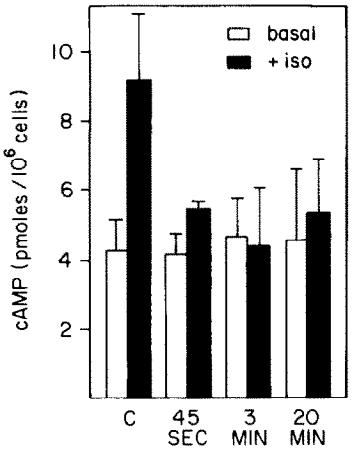

Fig. 2. Desensitization of lymphocytes by epinephrine at 4°. Cells (10 million per ml) were incubated in Krebs–Ringer bicarbonate buffer at 4° in the presence of 1 μM epinephrine or in its absence as a control (C). At the indicated time intervals, aliquots were diluted with 10 vol. of ice-cold epinephrine-free buffer and concentrated centrifugally (1500 g, 10 min). Cells were washed twice as described in the legend of Fig. 1, and then resuspended in fresh (epinephrine-free) buffer and incubated at 37° for 30 min. Aliquots were withdrawn for determination of isoproterenol (ISO) sensitivity as described in Methods. Results are the means ± S.D. of three separate determinations and representative of those obtained with three different preparations.

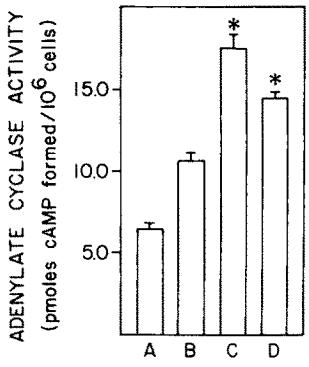

Fig. 3. Lymphocyte adenylate cyclase activation by pretreatment with guanyl nucleotide and β-adrenergic catecholamine agonists. Lymphocytes were incubated at 4° with no additions (A), 100 μM GMP.P(NH)P (B), and 100 μM GMP.P(NH)P with 100 μM isoproterenol (C) or 100 μM epinephrine (D) for 10 min and centrifugally washed as described in Methods. Results are the means ± S.D. of four separate determinations. Key: (*) significantly ($P < 0.01$) greater than pretreatment with GMP.P(NH)P alone.

Cells were incubated with [^{3}H]epinephrine under the same pretreatment conditions to quantify hormone bindings at 4° with inconsistent results. No binding which survived subsequent washing occurred before 30 min, at which time 300 molecules were bound per cell with high affinity when this was determined by Scatchard analysis ($K_A = 4 \times 10^{10}\ M^{-1}$, not shown). The bound radioactivity was sensitive to digestion with monoamine oxidase (1 mg/ml, 20 min at 30°, not shown).

Discussion

Our results indicate that brief exposure of some human tissues to β-adrenergic catecholamines is sufficient to initiate the desensitization process, probably reflecting the grave consequences of over-exposure to this potent class of agonists. Desensitization proceeded in the face of rapid degradation of hormone at 37°. Moreover, the desensitizing effects of epinephrine could be achieved by pretreating cells at 4°. Attempts to desensitize rodent astrocytoma cells by pretreatment with isoproterenol at 4° were unsuccessful [13], despite evidence that agonist (but not antagonist) binding by β-adrenergic receptors is of a higher affinity at that temperature compared to 37° [14, 15]. The rate of desensitization at 37° was slow in these cultured cells, however [16]. The more rapid rate at which desensitization occurred in freshly isolated human lymphocytes and its rapid induction at the low temperature suggest a different underlying mechanism of agonist-induced refractoriness.

Since desensitization occurred as a consequence of exposing cells to epinephrine for even brief periods at 4°, it appeared that only formation of the hormone–receptor complex might be prerequisite for desensitization. Formation of a hormone–receptor complex at the low temperature which survived the subsequent washing steps was indicated by the effect epinephrine and isoproterenol had on adenylate cyclase activation by pretreatment with

GMP.P(NH)P. Little of the stimulatory effect that epinephrine had on irreversible activation by GMP.P(NH)P could be attributed to hormone which was non-specifically bound and inadvertently transferred to the enzyme assay. At 4°, little [^{3}H]epinephrine was bound in the short times required for activation by GMP.P(NH)P and the hormone. After 30 min at the low pretreatment temperature, only ~300 molecules were bound per cell.

Despite the affinity ($K_A = 4 \times 10^{10}\,M^{-1}$), there was little to suggest that the radioligand was "specifically bound" by the cell surface β-adrenergic receptors. Monoamine oxidase sensitivity of binding suggested, however, that the tritiated ethylamine moiety of epinephrine, and perhaps the hormone itself, was still intact. If all the bound hormone had been readily dissociated, the maximum concentration of epinephrine in the enzyme assay would have approached 3 pM, a concentration whose effect on adenylate cyclase in the presence or absence of GMP.P(NH)P is imperceptible. Formation of an active hormone–receptor complex, therefore, occurred readily at 4°. Incubating the enzyme for up to 45 min at 4° or higher (23°) temperatures with GMP.P(NH)P and the agonists did not result in desensitization (not shown), however, suggesting that cellular integrity was requisite for desensitization in addition to formation of the receptor–hormone complex.

We found previously that desensitization of lymphocytes by isoproterenol *in vitro* occurred at agonist concentrations at which few receptors had to be occupied to initiate desensitization [3]. In the experiments described here, treatment with the endogenous β-adrenergic catecholamine epinephrine *in vitro*, even for very brief periods, was associated with the same loss of sensitivity, further indicating that a sensitive mechanism functions at the cellular level to protect target tissues from overexposure to catecholamines in man.

In summary, freshly isolated human lymphocytes incubated in Krebs–Ringer bicarbonate buffer with epinephrine underwent a time-dependent loss of sensitivity to β-adrenergic catecholamines determined as isoproterenol-dependent cAMP production by pretreated and washed cells. Under these pretreatment conditions, the rate of degradation of epinephrine was faster than the rate of desensitization. Pretreatment for as little as 45 sec at 4° followed by dilution in agonist-free buffer and washing resulted in desensitization when the cells were subsequently incubated at 37°. Our results suggest that desensitization under some conditions need not be due to the protracted exposure of target cells to agonists, but can be the consequence of the initial interaction of agonists with their specific cell surface receptors.

Acknowledgement—These investigations were supported by Veterans Administration funds.

The Biochemical Pharmacology Laboratory
Department of Medicine (UCLA-SFVP)
Veterans Administration Medical Center
Sepulveda, CA 91343, *U.S.A.*

J. FREDERICK KRALL*
MARIANNE CONNELLY
MICHAEL L. TUCK

REFERENCES

1. M. E. Connolly and J. K. Greenacre, *J. clin. Invest.* **58**, 1307 (1976).
2. H. G. Morris, S. A. Pusnak, J. C. Selner, K. Barzens and J. Barnes, *J. Cyclic Nucleotide Res.* **3**, 439 (1977).
3. J. F. Krall, M. Connelly and M. L. Tuck, *J. Pharmac. exp. Ther.* **214**, 554 (1980).
4. J. Mickey, R. Tate and R. J. Lefkowitz, *J. biol. Chem.* **250**, 5727 (1975).
5. C. Mukherjee and R. J. Lefkowtiz, *Proc. natn. Acad. Sci. U.S.A.* **73**, 1494 (1976).
6. P. J. Lefkowitz, D. Mullinkin and L. T. Williams, *Molec. Pharmac.* **14**, 376 (1978).
7. Y-P. Su, T. E. Harden and J. P. Perkins, *J. biol. Chem.* **254**, 38 (1979).
8. K. J. Catt, J. Harwood, C. Aguilera and M. L. Dufau, *Nature, Lond.* **280**, 109 (1979).
9. R. B. Hoffman, D. Mullikin-Kilpatrick and R. J. Lefkowitz, *J. Cyclic Nucleotide Res.* **5**, 355 (1979).
10. K. Burton, *Biochem. J.* **62**, 315 (1956).
11. J. F. Krall, M. Connelly, R. Weisbart and M. L. Tuck, *J. clin. Endocr. Metab.* **52**, 863 (1981).
12. J. D. Peuler and G. A. Johnson, *Life Sci.* **21**, 625 (1977).
13. G. A. Nickols and G. Brooker, *Proc. natn. Acad. Sci. U.S.A.* **75**, 5520 (1978).
14. P. A. Insel and M. Sanda, *J. biol. Chem.* **254**, 6554 (1979).
15. G. A. Weiland, K. P. Minneman and P. B. Molinoff, *Nature, Lond.* **281**, 114 (1979).
16. W. L. Terasaki, G. Brooker, J. deVellis, D. Inglish, C-Y. Hsu and R. D. Moylan, in *Advances in Cyclic Nucleotide Research* (Eds. W. J. George and L. J. Ignarro), Vol. 9, p. 33. Raven Press, New York (1978).

* Address all correspondence to: Dr. J. Frederick Krall, Biochemical Pharmacology Laboratory (111K), Veterans Administration Medical Center, 16111 Plummer St., Sepulveda, CA 91343, U.S.A.