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EFFECTS OF MERCAPTANS UPON DIHYDROPYRIDINE BINDING SITES ON TRANSVERSE TUBULES ISOLATED FROM TRIADS OF RABBIT SKELETAL MUSCLE

Neil R. Brandt, Richard M. Kawamoto and Anthony H. Caswell

Department of Pharmacology
University of Miami School of Medicine
P.O. Box 016189
Miami, Florida 33101

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The binding of nitrendipine to transverse (T) tubules isolated from skeletal muscle triads is inhibited by dithiothreitol (K_I ~0.05 mM) and glutathione (K_I~3 mM). The t 1/2's of inhibition (18.3 and 11.5 min, respectively) suggest that these hydrophylic reagents act upon the exposed surface of the vesicles. Dithiothreitol shifts the apparent K_D for nitrendipine from 8.5 nM to 30 nM without altering the B_{max} extrapolated by Scatchard analysis. That T-tubules isolated by disruption of triad junctions are constrained to have the protoplasmic (P) face uniformly exposed was experimentally confirmed. These studies show that a sulfhydryl residue on the P-face of the T-tubule influences the affinity of the receptor for dihydropyridines.

The membranes isolated from the transverse (T-) tubule system of skeletal muscle have the highest number of binding sites (B_{max} > 50 pmol/mg) for the Ca^{2+} channel antagonists known (1). We have previously demonstrated that these receptors are restricted to the T-tubules component of triad junctions found in skeletal muscle microsomes (B_{max} = 27 pmol/mg, K_D ~4 nM) (2). The affinity of these receptors for the dihydropyridines, however, is several orders of magnitude greater than the drug concentration required to block Ca^{2+} conductance in the intact muscle fibre (3). A similar discrepancy between the K_D for receptors on isolated cardiac sarcolemma (4) and the ID_{50} for plateau Ca^{2+} currents in papillary muscles has also been observed (5). Nevertheless, the physiological ED_{50} is identical to the K_D for receptors on membranes isolated from vascular smooth muscle (6, 7).

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This high physiological affinity may underlie the tissue selectivity of the dihydropyridines *in vivo*.

The discrepancy between the K_D and ED_{50} in skeletal muscle may arise by changes in the state of the receptor occurring during membrane isolation procedures. Dihydropyridines bind preferentially to inactive Ca^{2+} channels on cardiac membranes (8) and transitions from the low affinity (open and closed) states to high affinity (inactive) state may be induced by membrane dephosphorylation (6) and/or by loss of membrane potential (8). Here we report that the affinity of receptors for nitrendipine is decreased by treatment of T-tubules with mercaptans, suggesting that the receptor is sensitive to the oxidation state of a critical cysteine residue.

METHODS

Transverse tubules were isolated by mechanical disruption of skeletal muscle triad junctions as described by Lau et al (9). Protein was determined by the method of Bradford (10) with bovine serum albumin as standard. Nitrendipine binding was assayed by filtration on Whatman GF/C filters. Ligand concentrations, incubation times and reaction media are described in the figure legends. All filters were washed with 3 X 5 ml of ice cold reaction medium. Nonspecific binding was determined in the presence of excess unlabelled nisoldipine (a gift from A. Scriabine, Miles Institute for Preclinical Studies). The binding of [^{3}H]digoxin (50 nM) were performed under conditions identical to those of Lau et al (11). Nonspecific binding was determined in the presence of excess (5 μM) unlabelled digoxin. [^{3}H]nitrendipine (76 Ci/mmol) and [^{3}H]ouabain (18 Ci/mmol) were purchased from New England Nuclear. [^{3}H]digoxin (12.5 Ci/mmol) was purchased from Amersham. Dithiothreitol (DTT), glutathione (GSH) and oxidized glutathione (GSSG) were purchased from Sigma. All solutions were buffered to pH 7.3.

RESULTS

Dithiothreitol is a potent inhibitor (K_I ~ 0.05 mM) of nitrendipine binding to T-tubules suspended in osmotically buffered medium (Fig. 1). The binding is also inhibited by millimolar concentrations of glutathione (K_I ~ 3 mM). Decreased binding was observed at higher concentrations of oxidized glutathione. Solutions of the hexapeptide above 10 mM, however, become increasingly viscous and the apparent inhibition may be nonspecific. Dithiothreitol is expected to be more potent because of its higher redox potential (E'_o, pH.7.0 = -0.33V vs -0.24V for glutathione), and because it

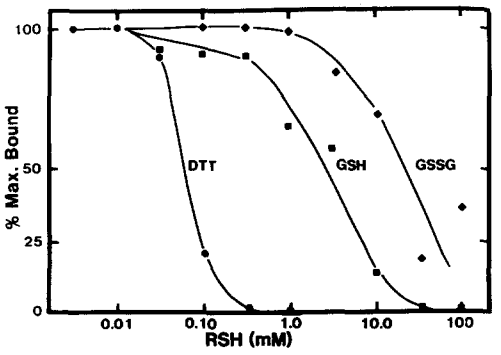

Fig. 1. Inhibition of specific nitrendipine binding by mercaptans. Transverse tubules (39 μg) were incubated in 200 mM sucrose, 20 mM Tris MOPS, pH 7.3, 4.2 nM [^{3}H]nitrendipine and dithiothreitol (DTT), glutathione (GSH) or oxidized glutathione (GSSG) at the concentrations indicated. Reaction volume was 1 ml and incubation time was 60 min at 23°. Nonspecific binding determined in the presence of 250 nM nisoldipine was less than 10% of the total bound in the absence of mercaptans and was subtracted before calculation of % maximum binding.

cyclizes to form an intramolecular disulfide rather than forming mixed disulfides with a protein residue (12). A similar concentration dependency of inhibition by the mercaptans of beta adrenergic antagonist binding to intact C_6 glioma cells has been reported (13).

Fig. 2 shows the effect of varying the times of incubation of T-tubules in dithiothreitol or glutathione before addition of [^{3}H]nitrendipine. The

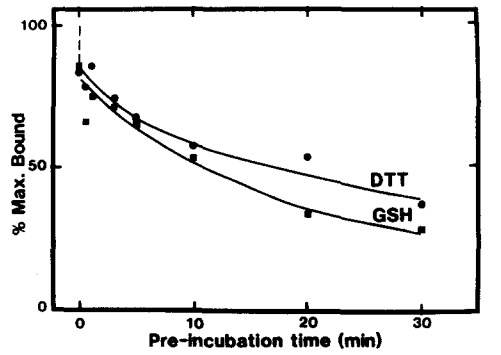

Fig. 2. Effect of time of mercaptan preincubation on specific nitrendipine binding to transverse tubules. Transverse tubules (50-60 μg) were suspended in 1 ml of 250 mM sucrose, 20 mM Tris MOPS, pH 7.3, and 5 mM dithiothreitol (●) or 10 mM glutathione (■) at 23°. After the preincubation time indicated, [^{3}H]nitrendipine was added to a final concentration of 4.2 nM. The binding reaction was quenched 1 min later by addition of 5 ml ice cold reaction buffer and immediate filtration. Zero preincubation binding was assayed by addition of transverse tubules to medium containing both [^{3}H]nitrendipine and the mercaptan. In the absence of added thiols, T-tubules bound 6.48$\pm$ 0.16 pmol nitrendipine/mg protein in 1 min. Non specific binding determined in the presence of 250 nM nisoldipine was less that 13% of total binding in the absence of mercaptans and was subtracted before calculation of % maximum binding.

transverse tubules were suspended in isosmotic medium and the reaction time with the radioactive ligand was limited to 1 min. A 20% decrease from untreated T-tubules was detected when the organelles were added to solutions containing both the mercaptan and nitrendipine. The remaining decrease was logarithmic with a $t_{1/2}$ for the overall reaction of 11.5 min for glutathione and 18.3 min for dithiothreitol. Nitrendipine was not displaced from the membranes when the reagents were added immediately before filtration. The absorption and emission spectra of the dihydropyridine in free solution were not altered upon addition of the mercaptans.

Dithiothreitol caused a rightward shift in the equilibrium binding curve for nitrendipine (Figure 3). Presoaking filters in 0.05% polyethylenimine (11) allowed extension of the experimental range to the solubility limit of the competing ligand, nisoldipine, in hypotonic medium. Scatchard analysis (inset) indicated a shift in the dissociation constant

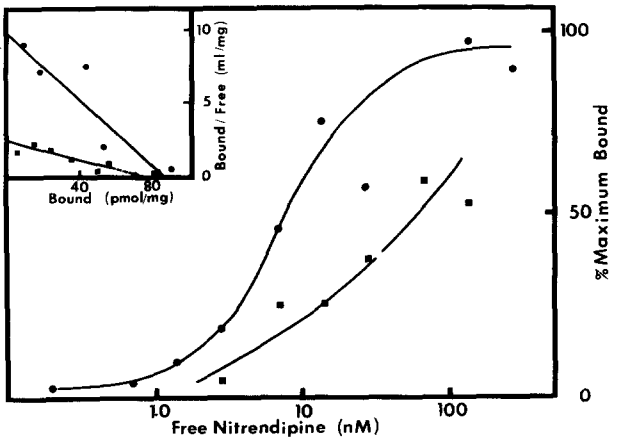

Fig. 3. Effect of dithiothreitol upon specific nitrendipine binding to T-tubules. Transverse tubules (30 μg) were suspended in 50 mM Tris Cl, pH 7.3 in the absence (●) and presence (■) of 5 mM dithiothreitol and varying concentrations of [^{3}H]nitrendipine. After incubation for 1 hr at 23°, binding reactions were assayed by filtration through GF/C membranes pretreated with 0.05% polyethylenimine to reduce nonspecific adsorption. Filters were washed 3 times with ice-cold 200 mM choline Cl, 20 mM Tris Cl, pH 7.3. Free nitrendipine was calculated as the difference between total nitrendipine in the binding mixture and the amount of ligand bound to the membranes. Nonspecific binding was assayed in the presence of 1 μM nisoldipine at low [^{3}H]nitrendipine levels and was less that 15% of total binding. Nonspecific binding assayed in the presence of 10 μM nisoldipine was 37% of total at 140 nM [^{3}H]nitrendipine and 60% of total at 280 mM [^{3}H]nitrendipine. These values were subtracted before calculation of % maximum binding. Inset: Scatchard plot for the binding in the absence (●) and presence (■) of dithiothreitol.

from 8.5 to 31 nM with the plots extrapolating to similar B_{max}'s (85 and 73 pmol/mg, respectively). These observations suggested that the effect of dithiothreitol was predominantly upon the binding affinity. This contention is supported by the observation that the data from the binding in the presence and absence of dithiothreitol fall on a single line in a Hill plot with $n_H = 0.82$. The validity of such extrapolations of the data to obtain B_{max} in the absence of observed saturation has been recently questioned (15). Nevertheless, it did not prove feasible to evaluate binding at concentrations above 200 nM nitrendipine owing to the high nonspecific association of nitrendipine with the filter and the organelles.

T-tubules can be isolated by mechanical disruption of skeletal muscle triads with retention of morphology (9). Lau et al (11) demonstrated that the number of ouabain binding sites increased 10-fold when the vesicles were incubated with 0.45 mg Na deoxycholate/mg protein. The binding of cardiac glycosides on the extracellular (E) surface of membranes requires the activation of the Na pump with ATP on the protoplasmic (P) surface. We have exploited the fact that ATP and ouabain are membrane impermeable while digoxin is permeable to estimate the orientation and exposure of T-tubular membranes. Table 1 shows that addition of SDS has no significant effect on the number of specific digoxin sites bound at the approximate K_D for the ligand. This indicates that ATP is accessible to all sites in the absence of detergent and hence no detectable fraction of vesicles are oriented with E face exposed (and P face latent). Addition of unlabelled ouabain decreased the digoxin sites bound from 12.3 to 9.7 pmol/mg indicating that 20% of vesicles are leaky with P and E faces exposed. SDS reduced the number of digoxin sites accessible to ouabain to 2.7 pmol/mg indicating that the detergent has exposed the E face of most of the vesicles. The number of ouabain sites bound was enhanced by detergent from 0.64 to 11.9 pmol/mg showing that 5% of the vesicles are leaky (P + E exposed). This latter value is more accurate than that calculated with digoxin since the latter ligand gives more non-specific binding owing to its higher lipid solubility.

Table 1. Cardiac glycoside binding to isolated T-tubules

	Amount Bound (pmol/mg)			
	Untreated		+ SDS	
Ligands	Specific	Nonspecific	Specific	Nonspecific
[^{3}H]digoxin (50 nM)	12.3 ± 1.1 (P, P + E)	9.7 ± 0.5	12.0 ± 0.8 (P, E, P + E)	9.9 ± 0.4
[^{3}H]digoxin (50 nM) + 5 μM ouabain	9.7 ± 1.0 (P)	9.7 ± 0.5	2.7 ± 1.0	9.9 ± 0.4
[^{3}H]ouabain (54 nM)	0.64 ± 0.05 (P + E)	0.42 ± 0.01	11.9 ± 0.3 (P, E, P + E)	0.46 ± 0.04

Transverse tubules (200 μg) were incubated in 1 ml binding medium (120 mM NaCl, 40 mM Tris Cl, pH 7.4, 10 mM MgATP and 1 mM EGTA) in the absence (untreated) or presence of 0.2 mg sodium dodecyl sulfate/mg protein (SDS) and the ligands listed in the table. Mixtures were equilibrated for 60 min at 37° then 0.3 ml aliquots were filtered through Millipore GSWP (0.22 μM) disks. The filters were washed 4 times with binding medium to which KCl (100 mM) and unlabelled ouabain (1 μM) were added. Non-specific binding was assayed in the presence of 5 μM digoxin and subtracted. All values are mean±SEM (n = 6). The orientation of the vesicles which bind the radioactive ligands are presented in parentheses under each assay (indicating exposure of faces which bind the ligand as discussed in the text).

Table I therefore shows that most T-tubules are sealed with the P face exposed. Hence leaky vesicles (P + E exposed) constituted 5-20% of the vesicles and we were unable to detect vesicles with E face exposed. This is consonant with our isolation of T-tubules from triads in which the morphology determines unambiguously that the P face is on the outside of the T-tubule vesicle.

DISCUSSION

We have shown that dithiothreitol decreases the affinity of the putative calcium channel for nitrendipine without apparently altering the number of binding sites. This new finding has analogies in other receptor systems. A similar phenomenon has been reported for the beta-adrenergic receptor on C6 glioma cells (13). On the other hand, dithiothreitol decreases the number of beta 1 receptors without altering the affinity on turkey erythrocyte membranes (16). The high affinity of the putative channel for dihydropyridines in homogenized tissue may arise in part because of oxidation of

critical sulfhydryl groups during tissue homogenization, a reaction which has been observed during the isolation of several enzymes (17, 18). Changes in the free sulfhydryl-disulfide ratio have been shown to affect the direction and extent of carbon flux through glycolysis and gluconeogenesis, in cell free systems giving rise to the hypothesis that changes in disulfides may exert a physiological modulation of enzymic processes (19).

The verapamil receptor, which interacts allosterically with the dihydropyridine receptor and may be on a different protein subunit of the putative Ca^{2+} channel (14, 20) has been shown to be exposed on the cytoplasmic surface of guinea pig myocytes (21). The binding of verapamil to a membrane preparation enriched in skeletal muscle T-tubules was inhibited by p-chloromercurisulfonic acid and N-ethylmaleimide but not dithiothreitol (20). We have demonstrated that T-tubules isolated from triad junctions are morphologically constrained to have the protoplasmic face uniformly exposed. Dithiothreitol (12) and glutathione which are highly water soluble rapidly inhibit nitrendipine binding under conditions where ouabain does not permeate the membrane. These observations indicate that a critical sulfhydryl residue on the dihydropyridine receptor is accessible from the cytoplasm where it could be kept in a reduced state in the intact cell by physiological buffers such as glutathione (19). Oxidation of that group to form a disulfide bridge may be involved in control of the activity of the Ca^{2+} channel.

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REFERENCES

1. Fosset, M., Jaimovich, E., Delpont, E. and Lazdunski, M. (1983) J. Biochem 258 6086-6092.

2. Brandt, N.R., Kawamoto, R.M. and Caswell, A.H. (1985) Receptor Res. in press.

3. Almers, W. and McCleskey, E.W. (1984) J. Physiol (London) 353 585-608.

4. Sarmiento, J.G., Janis, R.A., Colvin, R.,A., Triggle, D.J. and Katz, A.M. (1983) J. Mol. Cell Cardiol. 15 135-137.

5. Lee, K.S. and Tsien, R.W. (1983) Nature 302 790-794.

6. Janis, R.A. and Triggle, D.J. (1983) J. Medical Chem 26 775-785.

7. Janis, R.A. and Scriabine, A. (1983) Biochem. Pharmacol 32 3499-3507.

8. Sanguinetti, M.C. and Kass, R.S. (1984) Circ Res 55 336-348.

9. Lau, Y.H., Caswell, A.H., and Brunschwig, J-P. (1977) J. Biol. Chem. 252 5565-5574.

10. Bradford, M.M. (1976) Anal Biochem 72 248-254.

11. Lau, Y.H., Caswell, A.H., Garcia, M. and Lettelier, L. (1979) J. Gen. Physiol 74 335-349.

12. Cleland, W.W. (1964) Biochem 3 480-485.

13. Lucas, M., Hanoune, J. and Bockaert, J. (1978) Molec Pharmacol 14 227-236.

14. Galizzi, J-P., Fosset, M. and Lazdunski, M. (1984) B.B.R.C. 118 239-245.

15. Klotz, I.M. (1982) Science 217 1247-1249.

16. Vauguelin, G., Bottari, S., Andre, C., Jacobsson, B. and Strosberg, A.D. (1980) P.N.A.S. 77 3801-3805.

17. Meister, A. and Anderson, M.E. (1983) Ann Rev Biochem 52 711-760.

18. Van der Kloot, W. (1969) Science 164 1294-1295.

19. Gilbert, H.F. (1982) J. Biol Chem 287 12086-12091.

20. Goll, A., Ferry, D.R. and Glossmann, H. (1984) Eur J. Biochem 141 177-186.

21. Hescheler, J., Pelzer, D., Trube, G. and Trautwein, W. (1982) Pflugers Arch 393 287-291.