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Benzodiazepines stimulate sodium ion transport in frog skin epithelium

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Benzodiazepine binding sites are present in a variety of non-neuronal tissues including the kidney where they are localized to distal nephron segments. It is postulated that renal binding sites are involved in modulating ion transport. This study examined the effects of two benzodiazepines on sodium transport in frog skin epithelium, a model system for sodium transport in renal collecting duct. Treatment of short-circuited frog skin with diazepam (a non-selective benzodiazepine agonist) stimulated amiloride-sensitive short-circuit current, reflecting stimulation of active sodium transport. The diazepam response was equally effective with either serosal or mucosal application of the drug. Maximal stimulation of the current ($42 \pm 8\%$) was achieved with 10 μM diazepam (serosal). Short-circuit current was similarly augmented by serosal or mucosal addition of Ro5-4864, a benzodiazepine agonist with selective activity at peripheral (non-neuronal) receptors. The natriferic response to diazepam was additive to that of vasopressin or cyclic AMP suggesting that the mode of action of benzodiazepines is probably distinct from the cyclic AMP pathway. Thus, frog skin appears to be a useful model to examine the epithelial effects of benzodiazepines. Whether stimulation of sodium transport, however, involves peripheral-type benzodiazepine receptors in this tissue requires further studies.

Introduction

The novel peripheral benzodiazepine (BZD) receptor has recently been identified in several non-neuronal tissues of a variety of species (for a review, see Ref. 1). Analysis of ligand structure-activity studies has demonstrated marked differences between these receptors and conventional central (neuronal) BZD receptors. For instance, Ro5-4864 (4′-chlordiazepam) binds specifically and with high affinity to the peripheral binding sites and exhibits minimal neurological effects [1,2]. Almost all behaviorally-active BZD derivatives bind only weakly to peripheral binding sites. Diazepam is rather unique in that it binds with relative high affinity to peripheral and central binding sites [1,3].

Of interest is the discovery that the kidney contains a very high density of peripheral BZD binding sites [4–10], mostly localized to the tubular epithelium of distal nephron segments. Renal BZD receptors have been described in the medullary and cortical thick ascending limb [5–7], the distal convoluted tubule [5,6] and the medullary and cortical collecting ducts [7]. The physiological significance of peripheral BZD receptors in the kidney and elsewhere remains largely unknown. However, peripheral BZD agonists have been reported to exert functional and growth changes in a variety of non-renal systems in in vitro studies

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[1,3]. Furthermore, it has been proposed that peripheral receptors may serve as target sites for putative endogenous ligands resulting in modulatory effects on cell function [1]. In the kidney, the epithelial localization of peripheral BZD binding sites may imply a potential role in the modulation of ion transport. We recently described inhibition by benzodiazepines of ouabain-sensitive oxygen consumption in rabbit medullary thick ascending limb [11], indicating inhibition of NaCl absorption in this nephron segment.

While it is not known if peripheral BZD receptors are present in frog skin, we used this tissue in the current study because it is an easily accessible, hormonally sensitive epithelium which is used extensively to gain insights into mechanisms of transport of water and solutes. In particular, frog skin is considered a model tight epithelium closely analogous in function to the sodium-transporting portions of the mammalian collecting duct system of the kidney [12]. Our data demonstrate that benzodiazepine derivatives exert direct effects on frog skin to stimulate active sodium transport.

Materials and Methods

Abdominal skins of female frogs (*Rana pipiens*, West Jersey Biological Farms, Wenonah, NJ) were excised after double pithing and were mounted between the two halves of a Lucite double chamber as previously described [13,14]. Hemiskins were adjacent areas of equal size (0.80 cm^2) from either side of the ventral midline of the same skin. This allowed the study of control and experimental hemiskins of the same preparation. The standard Ringer solution used as the tissue bathing medium had the following composition (in mM): NaCl, 110; $NaHCO_3$, 2.3; KCl, 3.4; NaH_2PO_4, 0.15; Na_2HPO_4, 0.9; $MgCl_2$, 2.0, $CaCl_2$, 0.9 and dextrose, 5 (pH = 7.5–7.6). Ten ml of this solution was added to each side of the hemiskins, continuously bubbled with air to provide stirring and oxygenation. The transepithelial electrical parameters were monitored as previously described [13,14]. The tissue was continuously short-circuited except for brief periods during which the potential difference was clamped to 10 mV. The resultant change in transepithelial current was used to calculate the transepithelial tissue resistance (R_T). A dual-pen chart recorder was used to monitor the short-circuit current (I_{sc}) and voltage deflections of both hemiskins. I_{sc} in this tissue is an index of net transepithelial sodium transport [12].

Diazepam and Ro5-4864 were a generous gift from Dr. Peter Sorter (Hoffmann-LaRoche, Nutley, NJ). Vasopressin (Pitressin) was obtained from Parke-Davis, 8-(*p*-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate (8-CPT-cAMP) from ICN Pharmaceuticals, and amiloride from Merck Sharp and Dohme.

All values are reported as means ± S.E. for the group. Student's *t*-test for paired analysis was used to compare between control and experimental periods in the same skin or between paired hemiskins. *P* values greater than 0.05 were considered not statistically significant (*P* = n.s.).

Results

Before test agents were added to the bathing fluid in chambers, 1–3 h were allowed to achieve

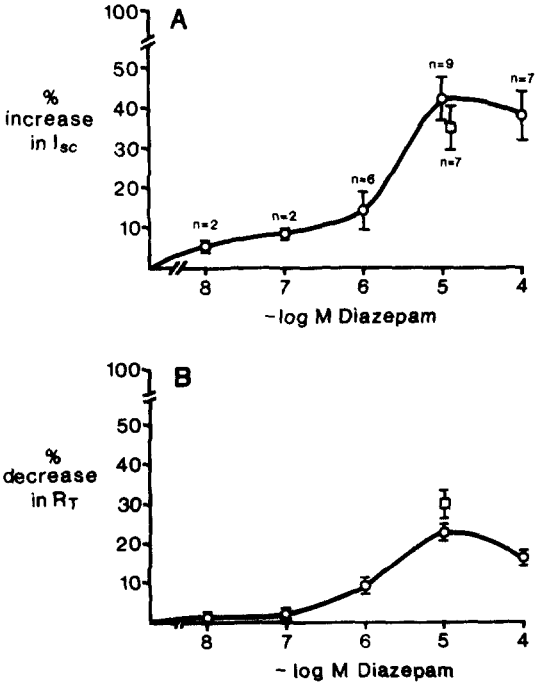

Fig. 1. Dose-response profile of the response of short-circuit current (I_{sc}) and total tissue resistance (R_T) to addition of diazepam. Values represent means ± S.E. (bars) with *n* as the number of skins in each group. Circles denote serosal, and squares mucosal application of diazepam.

stabilization of I_{sc} and R_T for at least 20 min. Diazepam added to the serosal solution of one hemiskin resulted in dose-dependent stimulation of I_{sc} (Fig. 1A) and a parallel decrease in R_T (Fig. 1B). Changes in electrical parameters were first observed within 5 min of diazepam addition. The maximal response was reached within 30 min after which time the effect was maintained for at least 1 h. Maximal response to serosal addition of diazepam was observed at 10 μM; I_{sc} increased by $42 \pm 8\%$ (from 27.1 ± 8.0 $\mu A/cm^2$ in control period to a peak value of 35.8 ± 9.6, $n = 9$, $P < 0.01$) and R_T decreased by $23 \pm 4\%$ (from 1128 ± 201 $\Omega \cdot cm^2$ to 843 ± 140, $P < 0.005$). The half maximal increase in I_{sc} is estimated at 3 μM diazepam. Treatment of the contralateral hemiskin with the vehicle for diazepam (ethanol or acetone at 0.20% and 0.15% v/v, respectively) resulted in no appreciable or persistent changes in I_{sc} or R_T. In an additional set of seven skins, the response to the mucosal addition of 10 μM diazepam was also tested. I_{sc} increased by $34 \pm 9\%$ (Fig. 1A) and R_T decreased by $29 \pm 4\%$ (Fig. 1B). On average, the magnitude and the time course of these effects were comparable to those observed with serosal addition of the drug. However, in any individual skin, the magnitude of the response to mucosal diazepam addition was variable, at times exceeding and at other times being lower than the response to serosal addition.

An additional group of four skins was studied with serosal addition of diazepam such that each skin was subjected sequentially to increasing doses between 10^{-8} and 10^{-4} M. A dose-response profile was obtained similar to that presented in Fig. 1 where one single dose was used in different groups of tissue. I_{sc} was augmented dose-dependently in each skin by $7 \pm 1\%$ (at 10^{-8} M), $14 \pm 2\%$ (at 10^{-7} M), $20 \pm 3\%$ (at 10^{-6} M), $39 \pm 3\%$ (at 10^{-5} M) and $43 \pm 4\%$ (at 10^{-4} M) when compared with initial (pretreatment) values.

To examine whether the increase in Isc reflected stimulation of sodium transport, the response to amiloride was tested (Fig. 2). In one group of hemiskins, diazepam (10 μM, serosal) was added first followed by amiloride (100 μM, mucosal) while the reverse sequence was tested in contralateral hemiskins. Amiloride abolished both basal as well as diazepam-stimulated I_{sc} in all

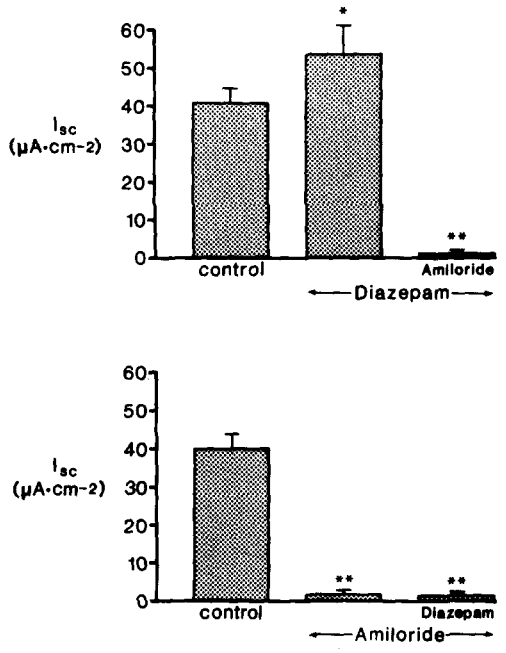

Fig. 2. Response of short-circuit current (I_{sc}) to sequential treatment of 5 pairs of frog hemiskins with diazepam (10 μM, serosal) and amiloride (100 μM, mucosal). In the upper panel, diazepam was added first followed by amiloride to one group of hemiskins. The reverse order was followed in the contralateral hemiskins and is depicted in the lower panel. Values are mean ± S.E. (bars); * $P < 0.05$ and ** $P < 0.005$ vs. control period.

tissues tested. Thus, the increase in I_{sc} induced by diazepam can be totally accounted for by stimulation of net sodium transport.

Fig. 3 depicts the effects on I_{sc} of serosal addition of Ro5-4864, a selective peripheral BZD agonist. At 10 μM, Ro5-4864 increased I_{sc} by $38 \pm 8\%$ ($n = 10$). The corresponding fall in R_T was $21 \pm 3\%$. In another set of skins (not shown), addition of 10 μM Ro5-4864 to the mucosal bath stimulated I_{sc} by $24 \pm 3\%$ and decreased R_T by $18 \pm 2\%$ ($n = 4$). These effects of Ro5-4864 are generally comparable to those of diazepam. The response to either benzodiazepine was readily reversible upon repeated washing of the skin with fresh Ringer's solution.

The natriferic response to diazepam was compared with that of vasopressin or cyclic AMP. Addition of vasopressin to the serosal bath at a supramaximal natriferic dose of 100 mU/ml [15] increased Isc to 45.5 ± 6.9 $\mu A/cm^2$ in control hemiskins ($n = 7$). In contralateral hemiskins pre-

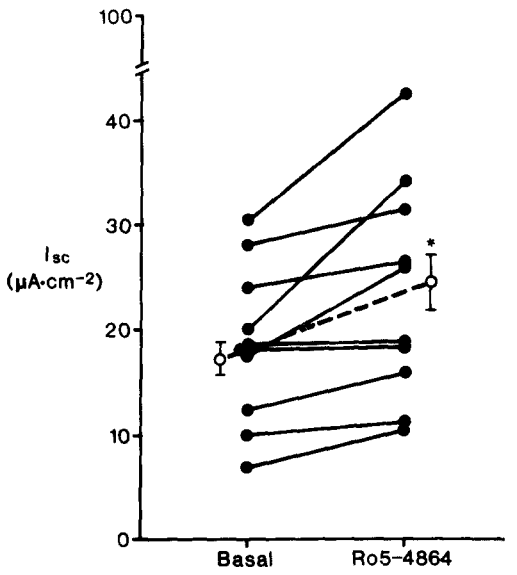

Fig. 3. Effect of Ro5-4864 (10 μM, serosal addition) on short-circuit current (I_{sc}) in frog skin ($n = 10$). Each line represents one skin. The dashed line connects the points (circles) representing mean ± S.E. (bars) for the group. * $P < 0.01$ vs. basal period.

treated with 10 μM diazepam (serosal plus mucosal addition), the subsequent addition of vasopressin increased I_{sc} to a comparable value (39.7 ± 6.2 $\mu A/cm^2$, P = n.s.). Similarly, addition of 100 μM 8-CPT-cAMP, a potent analogue of cyclic AMP [16], increased Isc to 48.8 ± 10 $\mu A/cm^2$ in control hemiskins ($n = 3$) and to a comparable value (45.3 ± 11 $\mu A/cm^2$, P = n.s.) in diazepam-pretreated contralateral hemiskins. The increase in Isc following vasopressin or 8-CPT-cAMP was at least 2-fold above baseline in contrast to the modest stimulation with diazepam (cf. Fig. 1A). In an additional set of skins pretreated with either vasopressin ($n = 3$) or 8-CPT-cAMP ($n = 3$), the subsequent addition of diazepam (10 μM) resulted in further significant increase in I_{sc}: from 48.0 ± 6 $\mu A/cm^2$ with vasopressin to 56.6 ± 6 ($P < 0.005$), and from 55.3 ± 3 $\mu A/cm^2$ with 8-CPT-cAMP to 66.3 ± 3 ($P < 0.01$). Taken together, these data indicate that the natriferic response to diazepam is less pronounced than that of vasopressin and cyclic AMP. Moreover, as the effects of diazepam appear to be additive to those of vasopressin and cyclic AMP, it may be concluded that the mode of action of diazepam is possibly distinct from that of the other agents.

Discussion

The results of this study demonstrate that in frog skin, I_{sc} is stimulated and R_T is concomitantly decreased upon addition of either diazepam, a non-selective BZD agonist, or Ro5-4864, a selective peripheral BZD agonist [1,2]. Amiloride blocks the increase in Isc after diazepam treatment, while pretreatment with amiloride prevents the characteristic stimulation of I_{sc} otherwise produced by diazepam. As amiloride in frog skin inhibits musocal sodium entry via specific sodium channels [17], these data indicate that diazepam-stimulated I_{sc} reflects an increase in net sodium transport. Moreover, since sodium transport is increased in the absence of an electrical or chemical gradient for sodium, it can be concluded that benzodiazepines stimulate active sodium transport in frog skin.

The response to diazepam and Ro5-4864 was demonstrable with either mucosal or serosal addition of either agent exhibiting no preferential sidedness. The response was equally prompt upon addition of the agent to either side of the skin. These results imply that the binding sites of BZD in frog skin which are required to initiate a natriferic response are present either in the mucosal as well as the serosal cell membrane or are located within an intracellular locus. It should be noted, however, that the presence of specific BZD binding sites in frog skin remains to be established. Nevertheless, the localization of peripheral BZD binding sites to the outer mitochondrial membrane in other tissues [18,19], is commensurate with the finding of nonpreferential sidedness of the natriferic effect of BZD agonists in frog skin and a possible intracellular binding site.

In principle, the stimulation of sodium transport can be viewed to involve at least one of several possible effects: (a) BZD derivatives could stimulate Na^+/K^+-ATPase directly, or indirectly through intracellular effects that lead to enhanced energy production or utilization for active transport; or (b) they could primarily stimulate amiloride-sensitive mucosal sodium entry into cells. Whichever is the mechanism, it does not appear that the response to diazepam is mediated through an increase in cellular cyclic AMP, a

major secondary messenger for vasopressin-mediated sodium transport in frog skin [15,20]. Our data indicate that the response to maximal natriferic doses of diazepam demonstrated appreciable additivity to the peak response of vasopressin or cyclic AMP analogue. In addition, the diazepam response was comparatively smaller. Thus, it is possible that BZD stimulation of sodium transport utilizes a minor natriferic pathway which appears to be distinct from that of cyclic AMP. Unfortunately, little is known regarding the mechanisms involved in this pathway. In fact, the mode of action of peripheral-type BZD agonists is currently unclear, in sharp contrast to the well known cellular effects of neuronally-active agonists (for details, see Ref. 1). It has been proposed, however, that activation of peripheral BZD receptors stimulates membrane-bound phospholipid methylation [21] or alternatively reduces cell membrane calcium entry [22]. Whether these or other mechanisms are involved in the response to BZD derivatives in frog skin awaits further study.

The results of the current study may shed some light on the potential role of BZD in epithelial transport. The recent discovery of a high density of peripheral-type BZD binding sites in several tubular segments of the mammalian distal nephron [5–7] may suggest that these binding sites may modulate tubular cell function. Previous studies have suggested an involvement of renal BZD binding sites in blood volume and pressure regulation based on alterations in the kinetics of ligand binding in a variety of experimental models. For instance, the density of BZD binding sites in the kidney were reported to be increased in deoxycorticosterone/salt hypertensive rats [23] and in Brattleboro rats with diabetes insipidus [24]. On the other hand, a decrease in renal binding sites has been found in spontaneously hypertensive rats [25] and in normal rats following adrenalectomy [26]. It is possible that in these models, renal tubular BZD receptors may play a contributing role in modulating epithelial transport. We recently reported that diazepam and Ro5-4864 diminish ouabain-sensitive oxygen consumption in suspensions of rabbit medullary thick-ascending limb tubules [11], implying inhibition of solute transport in this nephron segment. It is possible that within the kidney there exists a heterogeneity of response to BZD receptor activation presumably owing to the heterogeneity in structure and function of distal nephron segments.

Acknowledgments

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