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SHORT COMMUNICATIONS

Choline$^+$ is a low-affinity ligand for α_1-adrenoceptors

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Abstract—The effect of choline$^+$, a commonly used Na^+ substitute, on ligand binding to α_1-adrenoceptors was investigated. It was found that replacement of 25% of the Na^+ in a Krebs–Ringer bicarbonate buffer with choline$^+$ led to a 3-fold decrease in the apparent affinity of [^{3}H]prazosin for its binding site (i.e. the α_1-receptor) in a membrane preparation from brown adipose tissue, while no decrease in the total number of binding sites was observed. Similar effects were seen in membrane preparations from liver and brain. In competition experiments, it was found that choline$^+$ could inhibit [^{3}H]prazosin binding; from the inhibition curve, an affinity (K_i) of 31 mM choline$^+$ for the [^{3}H]prazosin-binding site could be calculated. In fully choline$^+$-substituted buffers, where the level of [^{3}H]prazosin binding was substantially reduced, both phentolamine and norepinephrine could still compete with [^{3}H]prazosin for its binding site, with virtually unaltered affinity; thus choline$^+$ did not substantially affect the characteristics of those receptors to which it did not bind. Choline$^+$ did not affect the binding characteristics of the β_1/β_2 radioligand [^{3}H]CGP-12177; thus, the effect on α_1-receptors was not due to general, unspecific effects on the membrane preparations. It is concluded that choline$^+$ possesses characteristics similar to those of a competitive ligand for the α_1-adrenoceptor; it has a low affinity but the competitive type of interaction of choline may nonetheless under experimental conditions interfere with agonist interaction with the α_1-receptor.

Key words: α_1-adrenergic receptors; [^{3}H]prazosin; choline; Na^+; norepinephrine; affinity

In several experimental systems, an interaction between α_1-adrenergic receptors and Na^+ ions has been demonstrated. Thus, the presence of Na^+ influences the affinity of adrenergic agents for the α_1-adrenoceptor [1–6], and the α_1-adrenergic coupling mechanism and the generation of second messengers would seem to be Na^+-dependent [7, 8]. α_1-Stimulation may regulate cellular processes involving Na^+, such as Na^+ influx into cells [9–11] and Na^+ efflux from cells (via stimulation of the Na^+/K^+-ATPase) [12–15]. Physiological processes such as renal handling of Na^+ are apparently under α_1-adrenergic control as well [16, 17]. Relationships between α_1-receptors and Na^+ have also been discussed in brown adipose tissue. This is true both for the coupling process [18, 19] and for the generation of second messenger signals [20, 21].

In studies dealing with the effect of Na^+ ions on physiological and biochemical processes, Na^+ depletion studies are often performed; i.e. Na^+ in the incubation medium is replaced by different Na^+ substitutes such as choline$^+$, *N*-methyl-D-glucamine$^+$ or Li^+. In connection with studies on the Na^+ dependence of α_1-adrenergic processes in brown adipose tissue, we observed inconsistencies between the effects of different Na^+ substitutes. This prompted us to examine whether any of the substitutes had a direct effect on the α_1-adrenergic receptor (as examined by [^{3}H]prazosin binding experiments), rather than being unable to replace Na^+ in the coupling or mediation process. We observed an apparent reduction in [^{3}H]prazosin binding to hamster brown adipose tissue in buffers containing choline$^+$.

In the experiments presented here we have analysed this phenomenon in order to define the mechanism of the choline$^+$ effect. The results demonstrate that the interaction of choline$^+$ with the α_1-receptor showed properties characteristic of a competitive ligand; however, the apparent affinity (K_i) was very low (≈31 mM). It is nevertheless evident that under certain experimental conditions, a substantial reduction in adrenergic responsiveness could be expected, solely due to this competitive effect of choline$^+$. This reduction could erroneously be interpreted as a demonstration of a Na^+ requirement for the mediation of α_1-adrenergic stimulation. Thus, the main consequence of the present observation of an interaction of choline$^+$ with the α_1-adrenoceptor would be found in the execution of experiments in which the significance of Na^+ for the mediation of α_1-adrenergic signals is examined.

Materials and Methods

Membrane preparations from brown adipose tissue. These were made from adult Syrian hamsters (*Mesocricetus auratus*) which had been living at 19–23° with food and water *ad lib.* Principally the method described by Mohell *et al.* [22] was used. The hamsters were killed by decapitation, and the interscapular, axillary and cervical brown adipose tissue was removed. The brown fat pieces were minced with scissors and homogenized at 0° in a Potter–Elvehjem homogenizer with a Teflon pestle in 10 mM Tris–HCl, 1 mM EDTA, 0.25 M sucrose, pH 7.4. The homogenate was filtered through silk cloth and the filtrate centrifuged for 30 min at 100,000 *g* at +4°. The pellet was rehomogenized in a small glass homogenizer with a Teflon pestle and washed three times by centrifugation as above. The final pellet was resuspended in Tris–Mg buffer (Tris–HCl 50 mM, $MgCl_2$ 10 mM, pH 7.4) at a protein concentration of about 10 mg/mL, frozen rapidly to −80° and stored until further use. Protein was determined by the method of Lowry *et al.* [23].

Measurement of [^{3}H]*prazosin and* [^{3}H]*CGP*-12177 *binding to membrane preparations.* The binding experiments were performed principally as described earlier [22]. In *equilibrium binding* studies, the membranes (1 mg protein/mL) were incubated in 1 mL of Krebs–Ringer bicarbonate buffer with the following standard composition (in mM): Na^+ 145, K^+ 6.0, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 128, SO_4^{2-} 1.2, HCO_3^- 25, $H_2PO_4^-$ 1.2, pH 7.4 [in the modified Krebs–Ringer bicarbonate buffers, NaCl and $NaHCO_3$ were replaced by the indicated amount of choline$^+$ Cl^- and, if necessary, choline$^+$ HCO_3^- (Sigma, St Louis, MO, U.S.A.)], together with different concentrations of tritiated ligand [[^{3}H]prazosin (Pfizer, Sandwich, U.K.) sp. act. 33 Ci/mmol (≈25 cpm/fmol) or [^{3}H]CGP-12177 (Amersham, U.K.), 42 Ci/mmol], in a shaking water bath at 37° for

15 min. The reaction was stopped with 2 × 1 mL ice-cold Tris–Mg buffer, and the incubation mixture immediately filtered through Whatman GF/C glass microfibre filters under vacuum. The filters were washed with 15 mL ice-cold buffer (diluted to 1/10), dried and their radioactivity determined in 5 mL of scintillation mixture [660 mL Triton X-100, 1340 mL toluene and 10 g PPO (2,5-diphenyloxazole)] in a liquid scintillation spectrometer. *Competition experiments* were performed as above, but with 1.7 nM [^{3}H]prazosin and various concentrations of choline$^+$ Cl^-, L-norepinephrine bitartrate (Sigma) or phentolamine methansulfonate (Ciba-Geigy, Basel, Switzerland) (the adrenergic ligands were diluted in 0.04% ascorbic acid in order to prevent oxidation). *Specific binding* was defined as the difference between total and nonspecific binding. When [^{3}H]prazosin binding was investigated, nonspecific binding was measured with a 5000-fold excess of phentolamine; it accounted for approx. 30% of the total binding at the [^{3}H]prazosin concentrations used (up to 3 nM). For [^{3}H]CGP-12177, nonspecific binding was measured with a 100-fold excess of L-alprenolol-D-tartrate (Sigma). The results of equilibrium binding studies were analysed according to Scatchard or with the reiterative general curve-fitting program of the KaleidaGraph data analysis/graphics application for Macintosh.

Results

Effect of choline$^+$ on [^{3}H]prazosin binding. Based on our preliminary results indicating choline$^+$ interaction with the α_1-adrenoceptor in brown adipose tissue, we performed equilibrium binding studies with [^{3}H]prazosin in membranes from this tissue. [^{3}H]Prazosin is well-characterized as a selective ligand for α_1-receptors in this tissue [22, 24, 25]. Two incubation media were used for these studies: a normal Krebs–Ringer bicarbonate buffer and a Krebs–Ringer bicarbonate buffer in which 25% of the Na^+ was substituted by choline$^+$ (this buffer thus contained 109 mM Na^+ and 36 mM choline$^+$).

As seen in Fig. 1A, replacement of 25% of the Na^+ with choline$^+$ did not change the nonspecific binding of [^{3}H]-prazosin (lower curves). However, it was clear from the total-binding curves that the binding parameters of [^{3}H]-prazosin were affected by the presence of choline$^+$. Analysis of the binding data, either directly from the binding curve (Fig. 1A) or from a Scatchard plot (Fig. 1B), showed that the apparent K_d value for specific [^{3}H]prazosin binding was increased when Na^+ was replaced with choline$^+$: from 0.27 ± 0.04 in normal Krebs–Ringer bicarbonate buffer to 0.96 ± 0.02 nM in the partially choline$^+$-substituted buffer ($N = 3$, $P < 0.01$, Student's paired t-test). However, the estimated total number of [^{3}H]prazosin binding sites (B_{max}) was unchanged by the substitution (about 25 fmol/mg protein). Thus, the interaction showed properties characteristic of a competitive interaction between choline$^+$ and [^{3}H]prazosin on the α_1-adrenergic receptor binding site.

An effect of choline$^+$ on [^{3}H]prazosin binding was also seen in isolated, intact brown-fat cells (not shown). This would be in agreement with an extracellular site of action of choline$^+$. In membrane preparations from liver and brain, the binding characteristics of [^{3}H]prazosin were changed by choline$^+$ in a manner similar to that observed in brown-adipose-tissue membranes (not shown); the effect was thus not tissue-specific.

Affinity of choline$^+$ for the [^{3}H]prazosin-binding site. In order to determine the affinity of choline$^+$ for the [^{3}H]-prazosin-binding site, we examined the ability of choline$^+$ to act as a competitor for [^{3}H]prazosin binding. We used Na^+ as a control for osmotic effects. We found that choline$^+$ was able to inhibit [^{3}H]prazosin binding (Fig. 2). Analysis of the inhibition kinetics indicated that the effect of choline$^+$ could be described as being competitive, with a K_i value of 31 ± 8 mM. Na^+ was also able to affect [^{3}H]prazosin binding although much higher concentrations were needed (K_i: 248 ± 57 mM). Thus, concentrations of Na^+ in the physiological range (≤145 mM) would hardly affect apparent [^{3}H]prazosin binding characteristics.

It may be noted that the reduction in the apparent K_d for [^{3}H]prazosin from ≈0.3 nM in normal buffer to ≈1.0 in a 36-mM-choline$^+$-containing buffer found above (Fig. 1) was in reasonably good agreement with a K_i for choline$^+$ of 31 mM (the expected shift in apparent K_d would be (36/31 + 1=) 2.2-fold, and the shift observed was ≈3-fold).

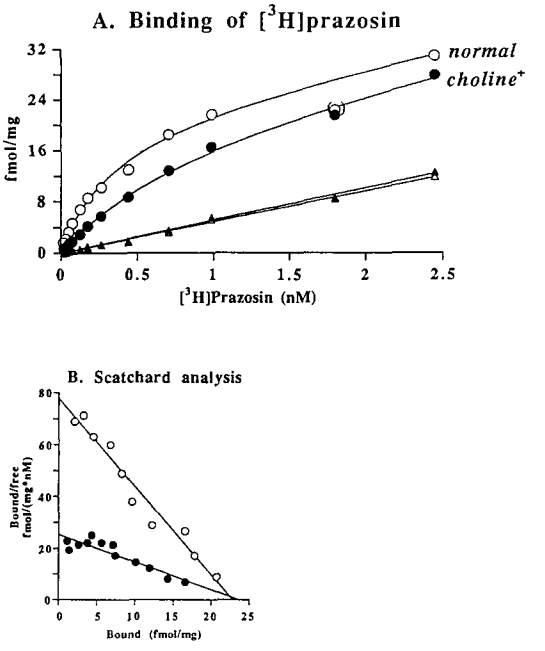

Fig. 1. Effect of choline$^+$ on [^{3}H]prazosin binding. (A) Equilibrium binding data of total (○, ●) and nonspecific (△, ▲) [^{3}H]prazosin binding in normal Krebs–Ringer bicarbonate buffer (open symbols) or in Krebs–Ringer bicarbonate buffer in which 25% (36 mM) of the Na^+ was replaced by choline$^+$ (filled symbols). Points are means of duplicate determinations in one membrane preparation. The mean data were analysed by the reiterative computer program for adherence to the formula $B_L = M \times L + B_{max} \times (L/K_d + L)$, where B_L is the total binding at a given ligand concentration L, M is the nonspecific binding constant, B_{max} is the total number of specific binding sites, and K_d is the apparent affinity for the ligand (here [^{3}H]prazosin). For all four data sets, M was calculated to be ≈5 (fmol/mg)/nM. For total binding in normal Krebs–Ringer bicarbonate buffer, B_{max} was calculated to be 21 fmol/mg and K_d to be 0.33 nM; in choline$^+$-substituted Krebs–Ringer bicarbonate buffer, the values were 21 and 0.96, respectively. Calculated values for B_{max} for the nonspecific-binding curves were infinitesimal. Pearson's correlation coefficients were greater than 0.997 in all cases. (B) The data for specific [^{3}H]prazosin binding obtained from A (except the indicated outlier), analysed according to Scatchard. The slopes ($= -1/K_d$) were determined by linear regression analysis; the values obtained were 0.29 nM in the normal Krebs–Ringer bicarbonate buffer and 0.93 nM in the choline$^+$-substituted buffer while the B_{max} was 23 fmol/mg protein in both cases. Pearson's correlation coefficient was 0.98 in normal buffer and 0.90 in choline$^+$-substituted buffer. The experiment shown is representative of three such experiments.

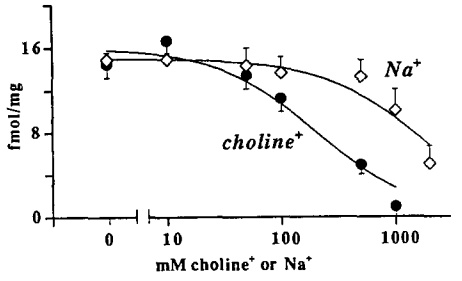

Fig. 2. Inhibition of [^{3}H]prazosin binding by choline$^+$ or Na^+. Membranes were incubated in Tris–Mg buffer with 1.7 nM [^{3}H]prazosin and the indicated concentrations of either choline$^+$ (●) or Na^+ (◇). The data points are means of three experiments (each performed in duplicate) on as many different membrane preparations. Specific [^{3}H]-prazosin binding is plotted as a function of choline$^+$ or Na^+ concentration. The data were analysed by the reiterative computer program for adherence to the formula $B_i = B_{max} \times L/(K_d(1 + I/K_i) + L)$, where B_i is the specific [^{3}H]prazosin binding observed at a given inhibitor concentration I, B_{max} is the total amount of specific [^{3}H]-prazosin-binding sites, L is the amount of [^{3}H]prazosin used (here 1.7 nM), K_d is the affinity of [^{3}H]prazosin for its site (here 0.3 nM (from Fig. 1) and K_i the apparent affinity of the inhibitor for the [^{3}H]prazosin-binding site. Data were weighted with the factor $1/SE^2$. K_i values obtained are discussed in the text. Pearson's correlation coefficients were 0.98 for both choline$^+$ and Na^+.

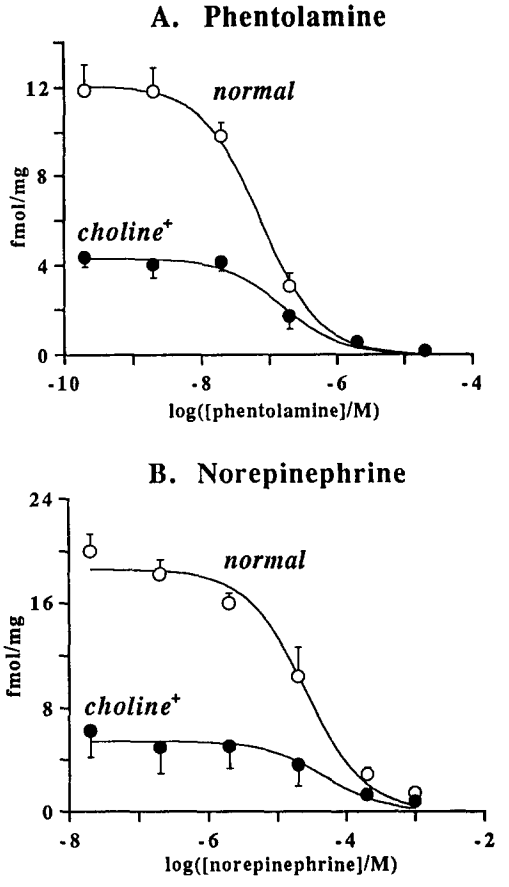

Fig. 3. Effect of choline$^+$ on antagonist and agonist affinities for α_1-receptors. Competition experiments were performed as described in Materials and Methods in normal Krebs–Ringer bicarbonate buffer (○) or Krebs–Ringer bicarbonate buffer in which 100% (145 mM) of the Na^+ had been substituted with choline$^+$ (●). (A) Phentolamine competition. The data are means and standard errors of five different membrane preparations, each examined in duplicate. Where not shown, the SE was smaller than the size of the symbol. The data were analysed by the reiterative computer program for adherence to the same formula as in Fig. 2. (B) As in A, except that norepinephrine was used as competitor. K_i values obtained are discussed in the text. Correlation coefficients were above 0.97 for all curves.

The demonstration in Fig. 1 of an unchanged total number of [^{3}H]prazosin-binding sites at the examined choline$^+$ concentration of 36 mM can thus probably be extended to indicate that a competitive type of interaction could be expected over the range of choline$^+$ concentrations of interest for physiological studies (i.e. up to total Na^+ substitution at 145 mM) (cf. the recent discussion by Leff and Dougall [26]). As expected, at high choline$^+$ concentrations, the [^{3}H]prazosin equilibrium binding curves experimentally obtained were shallow and difficult to evaluate with precision (not shown).

Antagonist and agonist competition of [^{3}H]*prazosin binding.* In order to investigate whether the presence of choline$^+$ affected the true affinity of the receptor (i.e. if any noncompetitive or uncompetitive effect of choline$^+$ contributed to the effect), we used adrenergic antagonists or agonists as competitors for the [^{3}H]prazosin-binding sites in the presence or absence of choline$^+$. If sites not occupied by choline$^+$ were unaffected by the presence of the choline$^+$ in the medium, the apparent affinity of the tested (ant)agonists for the "remaining" α_1-receptors (i.e. their K_i for competition of [^{3}H]prazosin binding) should be unaffected by the presence of choline$^+$ in the incubation medium. We used fully choline$^+$-substituted media (i.e. 145 mM choline$^+$) here, in comparison with normal medium (145 mM Na^+).

In agreement with the data in Fig. 2, the level of [^{3}H]-prazosin binding was reduced to approx. 30% in the fully choline$^+$-substituted buffer (Fig. 3A,B). However, the affinity (K_i) of the α-adrenergic antagonist phentolamine was not significantly affected, being nominally increased from 11 ± 1 to 24 ± 6 nM. Similarly, the affinity (K_i) of the α-adrenergic agonist norepinephrine was not significantly affected, being nominally increased from 4 ± 1 to 7 ± 3 μM. Thus, the affinity of the α_1-receptors for the adrenergic agents was not appreciably reduced in the presence of choline$^+$, in agreement with expectations if choline$^+$ interacts with the α_1-receptor with characteristics similar to those of a competitive inhibitor.

Specificity of choline$^+$ effects for α_1-receptors. In order to test if the effect of choline$^+$ was specific for α_1-receptors, we investigated the effect of choline$^+$ on the binding characteristics of β-adrenergic receptors. For these studies, we used the radioligand [^{3}H]CGP-12177 which binds with high affinity to β_1 and β_2 receptors in the tissue [24, 25, 27]. Scatchard analysis of [^{3}H]CGP-12177 equilibrium binding data showed that there was no difference between the binding characteristics in normal and 25%-choline$^+$-substituted buffer: both the affinity (0.84 ± 0.11 nM in normal and 0.87 ± 0.09 nM in choline$^+$-substituted buffer) and the total number of binding sites (B_{max}) (9.4 ± 0.6 fmol/mg protein vs 8.3 ± 0.2 fmol/mg protein) were unchanged (means ± SE from two experiments; not shown). This lack

of interaction of choline$^+$ with the β_1/β_2 receptor site is principally in agreement with earlier observations in other systems [28]. The absence of effect of choline$^+$ on the β-adrenoceptor also indicated that the effect of choline$^+$ on the α_1-receptor site was not unspecific, i.e. it was not due to a general detrimental effect of choline$^+$ on membranes, receptor proteins etc. Rather, the interaction of choline$^+$ specifically with α_1-receptors must be related to structural features distinguishing α_1-receptors from β-receptors.

Effect of other Na^+ *substitutes on* [^{3}H]*prazosin binding.* As indicated, not only choline$^+$, but also other cations, may be used as Na^+ substitutes in Na^+ depletion experiments. These other cations include *N*-methyl-D-glucamine$^+$. We observed no change in [^{3}H]prazosin binding characteristics with *N*-methyl-D-glucamine$^+$ added in Tris–Mg buffer or substituted into the Krebs–Ringer bicarbonate buffer (not shown). The choline$^+$ effect was thus not a Na^+ depletion effect but was directly related to the presence of choline$^+$ itself.

Discussion

In studies of ion dependence, Na^+ is often replaced by choline$^+$. We have here observed that choline$^+$ interacted with α_1-receptors, as demonstrated by changes in apparent [^{3}H]prazosin binding characteristics. We found that the interaction showed characteristics conforming to those expected of a competitive interaction, with an affinity of choline$^+$ for the α_1-receptor of approx. 31 mM. The choline effect was specific for α_1-adrenoceptors; i.e. it was not observed with β_1/β_2 adrenoceptors.

Choline$^+$ is not the only experimental agent which has been reported to interact with α_1-adrenergic receptors. Effects similar to those of choline$^+$ were observed by Wikberg and co-workers [29, 30] as effects of general anesthetics and organic solvents. These authors suggested, however, interaction of the agents with the lipid part of the membrane or conformational changes in the α_1-receptor as explanations for the effects observed. An ability of choline$^+$ to compete with norepinephrine for the carrier for neuronal uptake has been demonstrated earlier [31], indicating some structural similarity between the binding site on this carrier and the α_1-receptor.

Consequence of choline$^+$ *binding to the* α_1*-receptor for evaluation of experimental results.* In an experimental situation, the significance of the competitive interaction by choline$^+$ on the α_1-site is, of course, dependent on the concentrations of both choline$^+$ and agonist (e.g. norepinephrine) actually used. A full replacement with choline$^+$ of all Na^+ in a physiological saline-based buffer (which is a likely experimental situation in a Na^+ depletion study), corresponding to a choline$^+$ concentration of 145 mM, would lead to a (145/31 + 1=) ≈6-fold increase in the apparent EC_{50} for the agonist. Thus, if experiments are conducted with norepinephrine (or other α_1-agonists) at concentrations that are ≥100-fold the EC_{50} value, no residual influence of choline$^+$ would be expected; however, for lower concentrations of norepinephrine, it is the prediction of the present study that the presence of choline$^+$ could influence the results. For example, if experiments are performed at only three times the EC_{50} concentration, a decrease to about half of the response would be expected, solely due to the competitive action of choline$^+$ described here, and this could be erroneously interpreted as a demonstration of a Na^+ requirement for the mediation of α_1-adrenergic stimulation.

Thus, the main consequence of the present observation of an interaction of choline$^+$ with the α_1-adrenoceptor would be found in the practical execution of experiments in which the significance of Na^+ for the mediation of α_1-adrenergic signals is examined.

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