A Pharmacological Method to Estimate the \( pK_I \) of Competitive Inhibitors of Agonist Uptake Processes in Isolated Tissues

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Summary. An equation is derived from a mathematical model (proposed by Furchgott) which, under certain circumstances, estimates the \( pK_I \) (\(-\log \) dissociation constant) of a competitive inhibitor of agonist uptake by utilizing the sensitization of isolated tissues, to the substrate-agonist, by uptake inhibition. The method is theoretically more sound and appears to be improved by the use of potency-ratios of the substrate-agonist and an agonist which is not a substrate for the uptake process since this allows for the detection and correction of receptor and toxic effects of uptake inhibitors. The \( pK_I \) values of cocaine, desmethylimipramine and imipramine for the neuronal uptake of norepinephrine were estimated by this method in guinea-pig tracheae and left atria. Also, the \( pK_I \) values for 17-oestradiol, corticosterone, clonidine and metanephrine for the extraneuronal uptake of isoproterenol were estimated in guinea-pig tracheae (and cat left atria for 17-oestradiol). All estimates were consistent with literature \( pK_I \) values obtained biochemically with radiolabelled substrates.

Key words: Catecholamine uptake — Sensitization — \( pK_I \) — Estimation

Introduction

The sensitivity of isolated tissues to agonists that are actively removed from the region of the receptors by an uptake process (substrate-agonists) is often increased by inhibition of that uptake process (Trendelenburg 1963). Biochemical techniques, with radiolabelled substrates, have characterized many such uptake processes and quantitated the potency of competitive inhibitors for them by means of an estimated \( pK_I \) (\(-\log \) of the equilibrium dissociation constant of the antagonist for the site of uptake). However, the relationship, between the sensitivity of the organ to the substrate-agonist and the \( pK_I \) for uptake inhibition is, as yet, unclear.

A mathematical model, based on a model by Furchgott (1972), predicted the magnitude of the sensitization of guinea pig atria and tracheae to catecholamines, produced by inhibitors of neuronal and extraneuronal uptake, with reasonable accuracy (Kenakin 1980a). This present paper describes an equation, derived from that model, which yields an estimate of the \( pK_I \) for competitive inhibitors of uptake processes by utilizing the sensitization of tissues to substrate-agonists.

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Methods

Guinea-Pig Trachea. Guinea-pigs (Hartley, male, 300 — 350 g) were killed by cervical dislocation and the trachea was excised and placed in oxygenated (95\% O\(_2\)-5\% CO\(_2\)) Krebs solution of composition (in mM): Na\(^+\) 143, K\(^+\) 6, Ca\(^{2+}\) 2.5, Mg\(^{2+}\) 1.2, Cl\(^-\) 128, H\(_2\)PO\(_4\) 1.2, HCO\(_3\) 25, SO\(_4\) 1.2, d-glucose 11. The tracheae were trimmed of fat and cut into sections comprised of 2 rings of cartilage. Silk thread (5-0) was tied into indentations made on each side of the smooth muscle strip and the intervening cartilage was removed. The smooth muscle section was tied, under 1 g resting tension, to a perspex holder and to a Grass FT.03 isometric transducer. Contractions and relaxations were recorded on a Beckman R-511A dynograph. The bathing solution contained 100\(\mu\)M EDTA and 3\(\mu\)M phentolamine to protect against catecholamine oxidation (Hughes 1978) and activation of \(\alpha\)-adrenoceptors, respectively. Tissues were exposed to \((-)\)-isoproterenol (10\(\mu\)M) for 5 min, washed and left to equilibrate for 1 h during which they attained spontaneously, approximately 1 g tension above basal tension.

Concentration-response curves to either \((-)\)-norepinephrine or \((-)\)-isoproterenol and to salbutamol were obtained and the tissues equilibrated with various concentrations of inhibitors of neuronal or extraneuronal uptake for no less than 1 h. Concentration-response curves to the catecholamine and salbutamol were repeated in the presence of the uptake inhibitor.

Guinea-Pig Left Atria. The left atria of guinea-pigs (Hartley, male 300 — 350 g) were excised and clamped in a perspex holder such that the outer wall rested on a single platinum punctate electrode (Blinks 1965). The atria were placed in a heated (34\(^\circ\)C) 17 ml organ bath containing oxygenated (95\% O\(_2\)-5\% CO\(_2\)) Krebs-Henseleit solution of composition (in mM): Na\(^+\) 143, K\(^+\) 5.9, Ca\(^{2+}\) 2.6, Mg\(^{2+}\) 1.2, Cl\(^-\) 128, H\(_2\)PO\(_4\) 2.2, SO\(_4\) 1.2, HCO\(_3\) 25, d-glucose 10, and tied, under 0.5 g resting tension, to a Grass FT.03 isometric transducer. An electrical stimulus (square wave of 5 ms duration, 1 Hz, threshold voltage + 30\%) was delivered via the punctate and an external platinum electrode in the bathing solution and the resulting contractions were recorded on a Beckman R-511A dynagraph. Tissues were exposed to \((-)\)-isoproterenol (10\(\mu\)M) and washed for 1 h before commencement of experiments. Concentration-response curves to norepinephrine and salbutamol were obtained, the tissues washed, and exposed to various concentrations of inhibitors of neuronal uptake for 1 h. The responses to the agonists were then determined in the presence of the uptake inhibitors.

Cat Left Atria. Male cats (1 to 2 kg) were anaesthetized with an intraperitoneal injection of sodium pentobarbital (25 — 30 mg/kg), the hearts excised and placed in physiological salt solution of composition (in mM): Na\(^+\) 151, K\(^+\) 3.4, Ca\(^{2+}\) 2.5, Mg\(^{2+}\) 1.2, Cl\(^-\) 128.4, HCO\(_3\) 30, SO\(_4\) 1.2, H\(_2\)PO\(_4\) 1.0, d-glucose 5.5. The left atria were dissected and mounted like guinea-pig atria (see above) under a resting tension of 2 g. The tissues were stimulated at 2 Hz. The atria were exposed to isoproterenol (10 nM), washed for 1 h, and concentration-response curves to isoproterenol and salbutamol obtained. Responses to both agonists were redetermined after 1 h equilibrations with various concentrations of 17-oestradiol.

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Measurement of pr. In experiments with all tissues, the potentiations of responses were measured at the EC_{50} and the values of pr_{max} were determined in each tissue at 100 \times K_i. Small random changes in the sensitivity to salbutamol were not corrected for in the experiments with neuronal and extraneuronal uptake as they were not statistically significant (paired t-test on 4 EC_{50}). The exceptions were the data with metanephrine, which significantly altered the sensitivity of guinea-pig trachea to salbutamol.

Drugs Used. Agonists were (-)norepinephrine bitartrate (Sigma Chemical Co., St. Louis, USA), (-)isoproterenol HCI (Sigma Chemical Co., St. Louis, USA) and salbutamol (Schering Corp., FRG) all prepared in ascorbic acid (1 \mu M) and kept on ice throughout the experiments. Clonidine HCI (Boehringer-Ingelheim Co., FRG), cocaine HCI (Mallinckrodt Co., St. Louis, USA), (-)metanephrine HCI (Sigma Chemical Co., St. Louis, USA), deamethylimipramine HCI (US Vitamin), phenolamine HCI (CIBA-Geigy, Basel, Switzerland) and imipramine HCI (CIBA-Geigy, Basel, Switzerland) were all dissolved in ascorbic acid and kept on ice. Corticosterone (Sigma, Chemical Co., St. Louis, USA) and 17 \beta-oestradiol (Sigma Chemical Co., St. Louis, USA) were prepared in ethanol. The final concentration of ethanol never exceeded 0.2\% v/v in the organ bath.

Results

The Model

Assuming that the rate of entry of the substrate-agonist into the receptor compartment is governed by bulk diffusion and that uptake of the substrate-agonist can be described adequately by Michaelis-Menten kinetics, the following equation can be used to calculate the observed sensitization (x) of a tissue to a substrate-agonist (Kenakin 1980a):

\[ x = \frac{y(1 + [I]/K_i + [A]_b/K_{AR})}{y + [I]/K_i + [A]_b/K_{AR}} \]  

(1)

where [A]_b is the molar concentration of substrate agonist in the receptor compartment, K_{AR} is the equilibrium-dissociation constant of the substrate-agonist for the site of uptake, [I] is the molar concentration of uptake inhibitor and K_i is the equilibrium-dissociation constant of the uptake inhibitor for the site of uptake. The term x expresses sensitization as the multiple decrease in the concentration of agonist required to produce a given submaximal response in the presence of the uptake inhibitor and y refers to the maximal sensitization obtainable after uptake has been completely inhibited (Kenakin 1980a). Clearly, if the concentrations of substrate-agonist are near K_{AR} then uptake inhibitors would have little effect and sensitization of tissues would be too small to allow reliable estimation of the K_i by Eq. (1). Also, as can be seen from Eq. (1), x would depend upon the agonist-concentration and inhibitors of uptake would not produce parallel shifts to the left of concentration-response curves (Langer and Trendelenburg 1969). Therefore, this method can be used only when the concentration-response curves require concentrations of substrate-agonist below those that saturate uptake ([A]_b/K_{AR} \ll 1) such that inhibition of uptake produces parallel shifts to the left of concentration-response curves. Assuming [A]_b/K_{AR} \ll 1 and rearranging Eq. (1) into a logarithmic form, a linear regression can be obtained which yields log K_i as the abscissal intercept:

\[ \log \frac{y(x-1)}{y-x} = \log [I] - \log K_i. \]  

(2)

However, prerequisites to the effective use of Eq. (2) are reliable estimates of x and y. If the inhibitor of agonist uptake has post-synaptic action which decreases receptor sensitivity, little sensitization will be observed (Kenakin 1980a). Therefore, concurrent control experiments, with an agonist that is not a substrate for the uptake process, should be carried out to detect any actions of the uptake inhibitor, on tissue sensitivity, not related to the uptake process. The potency ratio of two such agonists would reflect the effect of uptake on the concentration-response curve to the substrate-agonist. The potency ratio (pr_j) after partial inhibition of uptake by I, would reflect sensitization to the substrate-agonist and the effects on the sensitivity to the non substrate-agonist therefore serving as a correction for post-synaptic effects. For example, if an inhibitor of uptake produced a 10-fold sensitization to an agonist-substrate but a two-fold shift to the right because of a receptor antagonism, the observed effect would be a five-fold sensitization. The two-fold shift to the right (receptor effect) would be detected with a non-substrate agonist. The net effect of the uptake inhibitor on the potency-ratio of the substrate and non-substrate agonist would be 10, the true effect of the uptake inhibitor on the uptake of the substrate-agonist. The pr_{max} is defined as the maximal difference in the potency-ratio of a substrate and non-substrate agonist and is the estimation of y, the maximal sensitization that would have been observed for the substrate-agonist if the uptake inhibitor were completely devoid of receptor-antagonist properties. Then Eq. (2) can be rewritten:

\[ \log \frac{pr_{max}(pr_j-1)}{pr_{max} - pr_j} = \log [I] - \log K_i. \]  

(3)

It should be recognized that a non-linear fitting of the anti-log form of Eq. (3) would most likely be the optimum method to estimate K_i but the logarithmic form is used here to allow convenient analysis by simple linear regression. There are three aspects of the regression according to this equation that should be considered in its’ routine use.

Firstly, since most likely more than one estimate of pr_j will be compared to a single parameter (pr_{max}) in this analysis, it is worth considering the effect of errors, on the regression, in pr_{max}. Figure 1 shows the behavior of the regression when pr_{max} is underestimated by 50\% or overestimated by 100\%.