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IONOPHORE MEDIATED CATECHOLAMINE RELEASE FROM CHROMAFFIN GRANULES

COMPARISON OF X-537 A AND X-14547 A EFFECTS

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Abstract—The carboxylic ionophore X-14547 A induces release of catecholamines from bovine chromaffin granules. This release is rapid and depends upon the ratio of ionophore to granule protein. When compared to X-537 A (lasolacid) which is known to mediate catecholamine release from the same preparation, X-14547 A is about 10 times more potent on a molar basis. Catecholamine release by X-14547 A is not accompanied by release of other components (ATP, protein or dopamine β-hydroxylase) of the granule matrix and therefore is not caused by lysis of the granule as previously proposed for X-537 A. X-14547 A also differs from X-537 A by its lack of specificity, both adrenaline and noradrenaline being equally released by this ionophore. These differences might reflect different release mechanisms. The absence of noradrenaline transport across a chloroform phase by X-14547 A suggests that its action might not involve direct translocation of the catecholamine cation across the granule membrane.

The antibiotic X-14547 A has been recently isolated from cultures of *Streptomyces antibioticus* (NRRL 8167) [1]. It is representative of the monocarboxylic ionophore group [2] which includes the Ca^{2+} ionophores X-537 A (lasolacid) and A-23187 (calcimycin). Preliminary studies have shown that at a pH higher than 8.0, X-14547 A is able to transport Ca^{2+} ions across a bulk chloroform phase separating two water phases ([3], and J. Bolte, C. Demuynck and G. Jeminet, unpublished observations). Like X-537 A, X-14547 A also forms complexes with the chiral primary amine 4-bromo-phenethylamine. However, X-ray studies [4, 5] have indicated differences in the structures of the two complexes. X-14547 A forms a 2:1 complex with a jaw-like conformation of the two ionophore molecules around the ammonium cation, whereas a 1:1 stoichiometry is observed with X-537 A [6]. The existence of such complexes suggests that X-14547 A, as well as X-537 A, could transport catecholamines across biological membranes [7–10].

Chromaffin granules contain a high concentration of catecholamines (0.7 M, [11]) which are part of a multicomponent high molecular weight complex [12] containing also Ca^{2+}, Mg^{2+}, ATP and proteins. Addition of X-537 A to a chromaffin granule suspension induces rapid release of the catecholamines by direct transport of the amine across the granule membrane [9, 10]. In the present communication, X-14547 A is shown to induce catecholamine release from chromaffin granules and this release is compared to that induced by X-537 A.

MATERIALS AND METHODS

Ionophores. X-14547 A has been isolated from *Streptomyces antibioticus* (NRRL 8167) by a procedure to be published in details elsewhere. The growth medium contained (w/v): glucose, 0.1%; beet molasses, 0.2%; soya meal, 0.05%; $CaCO_3$, 0.02%; yeast extract, 0.01%. The cultures were grown aerobically at 27° in a 20 litre fermentor stirred at 620 rpm. After 6 days mycelia were harvested by centrifugation and extracted twice with ethanol. The culture medium was extracted three times with ethyl acetate. The two organic solutions were pooled and concentrated. The residue was solubilised by 0.1 N HCl and extracted with ether. The brown extract was purified by chromatography on a Kieselgel 60 (Merck, Darmstadt, West Germany) column eluted by cyclohexane–diethyl ether and cyclohexane–ethyl acetate gradients. The physical properties of the isolated compound have been compared to these of an authentic sample of X-14547 A (gift of Dr. J. W. Westley) and found to be identical [5] by the following techniques: mass spectroscopy, polarimetry, i.r. and u.v. visible spectroscopy and ^1H and ^{13}C n.m.r.

X-537 A, Na^+ salt was purchased from Sigma Chemical Co. (St. Louis, MO) and was exchanged

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Abbreviations used: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; DBH, dopamine β-hydroxylase; DMSO, dimethyl sulphoxide.

to the corresponding free acid. A-23187 (calcimycin) was prepared as described [13].

Stock solutions (10 mg/ml) were made in ethanol (X-14547 A) or in DMSO/ethanol (1:1) (X-537 A and A 23187) and were diluted in ethanol. Ethanol and DMSO were present in the incubation mixtures at less than 1 and 0.4%, respectively and were adjusted at all ionophore concentrations. DMSO did not induce catecholamine release at concentrations up to 0.5%.

Chromaffin granule preparation. Bovine chromaffin granules were prepared by successive differential centrifugation at 27,000, 21,000 and 16,300 *g* for 30 min in 0.3 M sucrose, 10 mM NaOH–Hepes buffer (pH 7.0) [14]. This technique gave granule preparations containing 1.7 ± 0.6 μmole catecholamine/mg protein (mean ± S.E., $n = 16$), which appeared to be homogenous by electron microscopy.

Transport of noradrenaline across a chloroform phase. The experimental device was that of Pinkerton, Steiwauf and Dawkins [15] with mechanical stirring of the two aqueous phases. The aqueous solutions (4 ml) contained phosphate buffer and 10 mM noradrenaline and 2% ascorbic acid in the starting solution. The pH of the starting solution was varied from 6.0 to 8.0 whereas that of the receiving one was maintained at pH 7.0. The chloroform solution (10 ml) contained 0.4 mM ionophore. Noradrenaline transport was followed by fluorometric assay of catecholamine on 500 μl aliquots of the receiving solution [16].

Analytical techniques. (1) *Catecholamine assay.* Catecholamines were assayed essentially as described by Von Euler and Lishajko [16]. To obtain total catecholamine, the trihydroxyindole derivative was assayed fluorometrically (excitation 420 nm, emission 525 nm) with adrenaline standards. When a differential assay of noradrenaline and adrenaline was required, the fluorescence of the trihydroxyindole derivative was rapidly measured at λ excitation = 395 nm, λ emission = 490 nm and at λ excitation = 436 nm, λ emission = 540 nm [10]. The noradrenaline and adrenaline content was derived with a 9825 Hewlett–Packard computer from simultaneous equations, using measurements of the fluorescence under the same conditions of noradrenaline and adrenaline standards. (2) *ATP assay.* ATP was measured by firefly luciferin–luciferase reaction as in [17]. (3) *Dopamine β-hydroxylase activity assay.* The activity was assayed at pH 6.5 in 0.1 M phos-

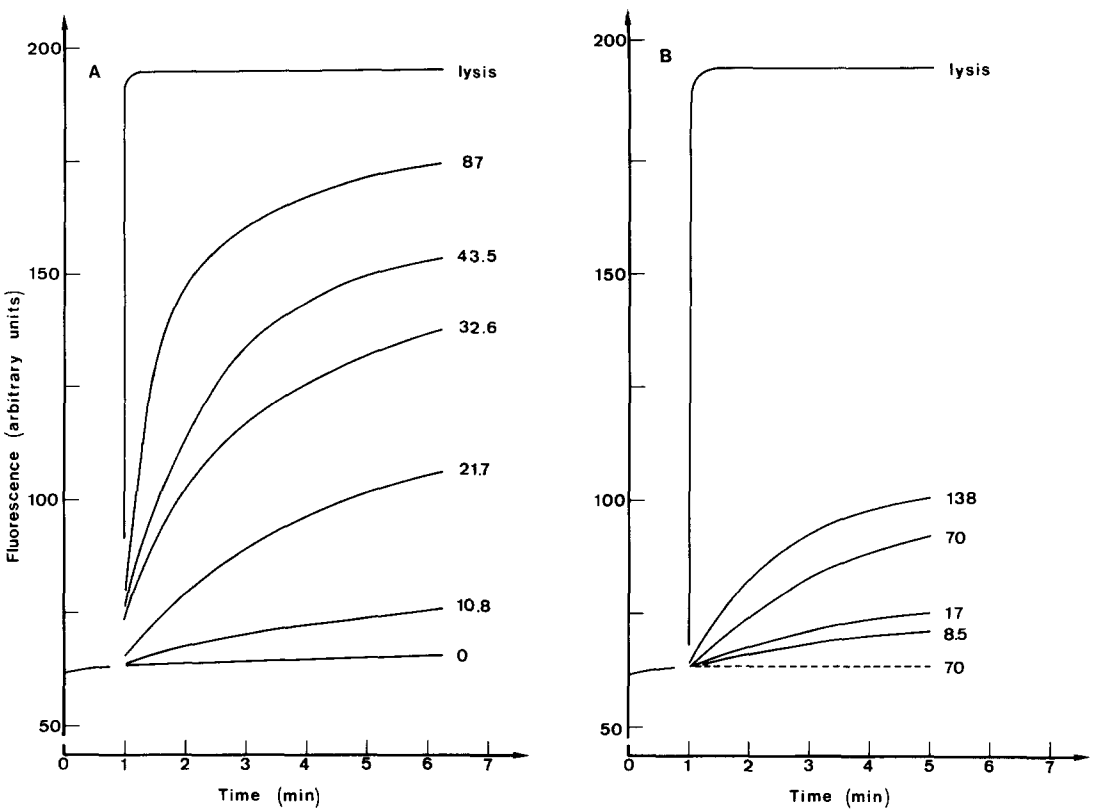

Fig. 1. Effect of X-14547 A, X-537 A and A 23187 on the release of chromaffin granule catecholamines. Chromaffin granules (0.46 mg protein) were incubated at 20° in 2 ml of 10 mM Hepes buffer (pH 7.0) containing 0.3 M sucrose. The release of catecholamine induced by ionophore addition was followed by monitoring fluorescence (excitation 280 nm, emission 320 nm) increase. The fluorescence increase corresponding to complete release of monoamines was determined by osmotic lysis of the chromaffin granules in 10 mM Hepes (pH 7.0). Results were corrected for ionophore absorbance at 280 nm by measuring the fluorescence of lysed granules at each ionophore concentration. Ionophore doses are in μg/mg of granule protein. (A) Effect of X-14547 A. (B) Effect of X-537 A (solid line) and A 23187 (dotted line).

phate buffer, with tyramine (10 mM) as a substrate, as described in [18, 19]. One activity unit corresponds to one nmole octopamine formed/min. (4) *Protein assay*. Proteins were estimated by the Lowry procedure with bovine serum albumin as a standard, following precipitation in 5% trichloroacetic acid and redissolution in 2% deoxycholate–3% NaOH.

Instrumentation. Fluorescence was measured with a JY 3 C Jobin–Yvon double monochromator fluorimeter.

RESULTS

Catecholamine efflux induced by carboxylic ionophores. The endogenous fluorescence of catecholamines has already been utilised to measure catecholamine efflux from chromaffin granules [20]. The method is based on the fact that the high concentration of intragranular monoamines results in a quenching of the catecholamine fluorescence [12]. Addition of X-14547 A to a chromaffin granule suspension induced an increase of fluorescence (excitation 280 nm, emission 320 nm) which was attributed to the release of catecholamines from their storage sites (Fig. 1A). The reaction was rapid and dose-dependent. At 87 μg ionophore/mg of granule protein more than 80% of the fluorescence increase induced by hypoosmotic lysis was observed in 5 min. A concentration of 2.5 μg X-14547 A/ml (10 μg ionophore/mg of granule protein) gave a detectable effect. In contrast, under the same conditions A 23187 was totally inactive and X-537 A showed a lower activity (Fig. 1B). Seventy μg X-537 A/mg of granule protein induced in 5 min only 20% of the fluorescence increase evoked by osmotic lysis.

With X-14547 A the rate of catecholamine efflux, measured as the fluorescence increase observed in the first minute following ionophore addition, increased linearly with the ionophore to protein ratio, up to 80 μg ionophore/mg of granule protein (Fig. 2). It should be noted that identical results were obtained when the ionophore to granule ratio was varied either at constant granule or at constant ionophore concentrations.

Specific release of catecholamines by X-14547 A. In isotonic sucrose medium, X-537 A has been reported to induce osmotic lysis of chromaffin granules as shown by the simultaneous release of various components of the granule matrix [10]. We confirmed this result, since treatment of chromaffin granules released large amounts of ATP, proteins and dopamine β-hydroxylase (DBH) activity which were present with catecholamines in the supernatant. To compare the amounts of the different substances released results obtained in the presence of the ionophore were expressed as percentage of those obtained by osmotic lysis and were corrected for values observed in the absence of ionophores (Fig. 3B). At the concentration of X-537 A which induced in 8 min 50% of the maximal catecholamine release, ATP, proteins and DBH activity were also found in the same proportion in the granule supernatant (55, 45 and 50% of their maximal release, respectively). This result supports the hypothesis that lysis occurs either as the mode or as the consequence of catecholamine release by X-537 A [10]. In contrast X-14547 A had only a limited lytic effect at a concentration range where it induced large catecholamine release (Fig. 3A). At 70 μg ionophore/mg protein, 75% of the endogenous catecholamines and only 20–25% of the ATP, protein and DBH activity

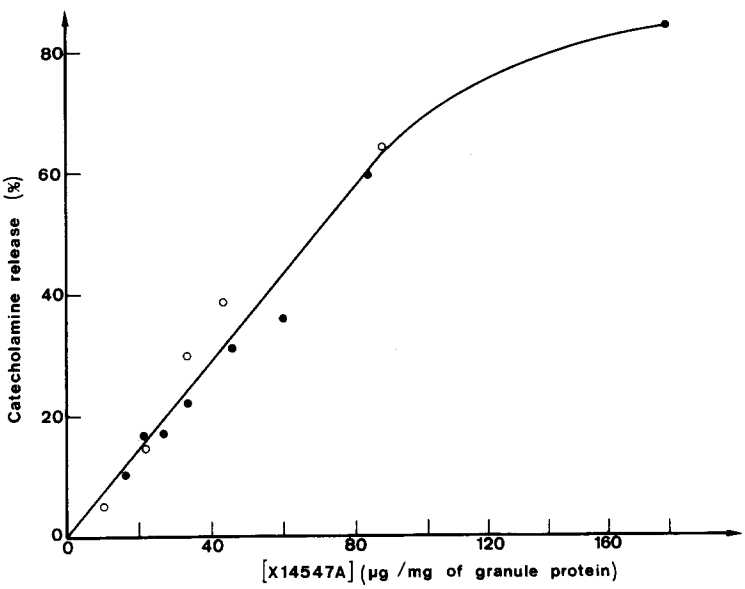

Fig. 2. Catecholamine release by X-14547 A as a function of the ionophore to granule protein ratio. (●), variation of the granule concentration at constant X-14547 A concentration (5 μg/ml); (○), variation of the ionophore concentration at constant granule concentration (0.23 mg protein/ml). Experimental conditions were as in Fig. 1. Release is calculated as the ratio of fluorescence increase observed one min after ionophore addition to that induced by osmotic lysis in the presence of the same ionophore concentration.

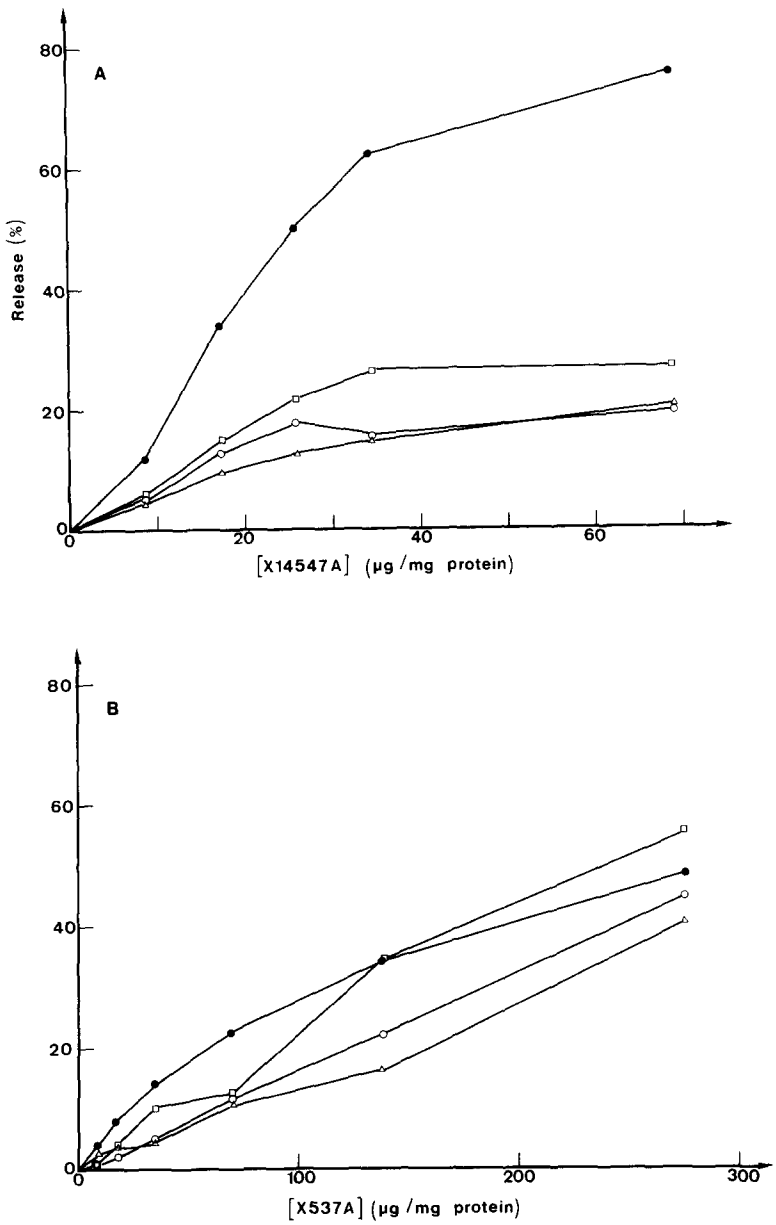

Fig. 3. Comparison of catecholamime, ATP, protein and DBH activity release by X-14547 A and X-537 A. Granules (0.58 mg protein) were incubated for 8 min at 20° in 2 ml of 10 mM Hepes (pH 7.0), 0.3 M sucrose containing variable amounts of either X-14547 A (A) or X-537 A (B). The suspensions were then centrifuged at 4° for 5 min at 47,000 *g* and the pellets resuspended in 1 ml of 10 mM Hepes (pH 7.0). Catecholamines (●), ATP (□), protein (○) and DBH activity (△) were measured in supernatant and pellet fractions. The amounts released were calculated from supernatant measurements and were expressed as percentage of controls lysed in 10 mM Hepes (pH 7.0). Hypoosmotic lysis released, per mg of initial granule protein: 1720 nmole catecholamine (100% of the initial store); 590 nmole ATP (100% of the store); 0.65 mg protein (65% of the store); 52 DBH units (40% of the store). Results were corrected for release in the absence of ionophore (less than 10% of that obtained by osmotic lysis).

were present in the supernatant. X-14547 A thus appears to release catecholamines without producing drastic granule lysis.

Differential release of catecholamines. Pellets and supernatants of chromaffin granules treated with the ionophores X-537 A and X-14547 A were analysed for their noradrenaline and adrenaline content (Fig. 4). With X-537 A, the supernatant of treated granules was characterised by a high noradrenaline content (60%). On the other hand, that of the corresponding pellets was low and decreased from 22 to 11% when the ionophore concentration was

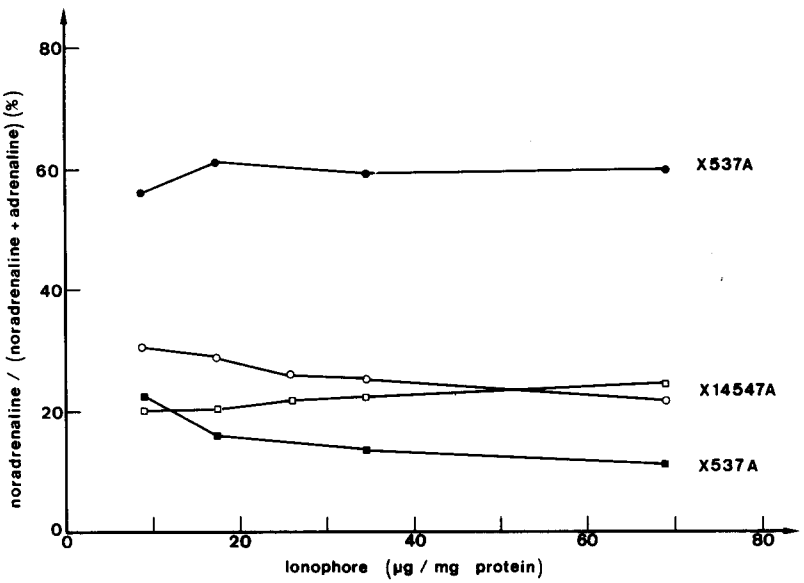

Fig. 4. Release of noradrenaline by X-14547 A and X-537 A. Experimental conditions are as in Fig. 3. Data are expressed as the relative noradrenaline content of each fraction: pellets (□, ■) and supernatants (○, ●) of granules treated either with X-14547 A (○, □) or X-537 A (●, ■). The release observed in absence of ionophore has been subtracted from supernatant values. The noradrenaline content of osmotically lysed granules was 23 ± 3% (S.E., $n = 3$).

increased from 10 to 70 μg/mg protein. This result supports previous investigations indicating that X-537 A translocates preferentially noradrenaline [10, 21, 22].

A different result was obtained when the same experiment was repeated with X-14547 A. With this compound, supernatants had the same ratio of noradrenaline to adrenaline as pellets for an ionophore concentration range inducing from 10 to 80% of the total catecholamine release (Fig. 4). This relative composition did not differ from that of untreated granules (23% noradrenaline, measured on the supernatant of osmotically lysed samples), thus suggesting that X-14547 A was not specific for a catecholamine. However, this experiment did not rule out the possibility that the ionophore might give a kinetic advantage to one species and would thus induce a more rapid efflux of this species. To investigate this possibility, the kinetics of the appearance in the supernatant of the two monoamines were measured (Fig. 5). Granules incubated for various times with the ionophore were filtered on Millipore filters and the supernatants were analysed for adrenaline and noradrenaline. Similar kinetics were obtained when each released amine was expressed as fraction of the corresponding untreated granule store.

Ionophore-mediated transport of noradrenaline across a chloroform phase. X-537 A has been reported to be able to transfer catecholamines across a layer of chloroform separating two sides of a U-shaped chamber containing two buffered aqueous phases [9]. Results of such an experiment performed with noradrenaline are shown in Table 1. When the same experiment was repeated with X-14547 A as the ionophore, no noradrenaline transport could be detected. Variation of the pH of the starting chamber from 6.0 to 8.0 did not change the result of the experiment.

DISCUSSION

The present communication shows that the monocarboxylic ionophore X-14547 A promotes rapid catecholamine release from chromaffin granules and that is more potent in this system than X-537 A (Fig. 1). The X-14547 A effect is dependent upon the ionophore to granule ratio (Fig. 2), as is generally observed with lipophilic drugs which have a high partition coefficient in phospholipids. At the same granule concentration X-14547 A was 9 times more effective in inducing 50% of catecholamine release (Fig. 3A and B). Two important differences in the mode of action of the two drugs were also noted: (i) catecholamine release by X-14547 A is not accompanied by granule lysis as in the case of X537 A (Fig. 3), and (ii) X-14547 A promotes the efflux of both adrenaline and noradrenaline with similar efficiency whereas X-537 A is apparently more specific for noradrenaline (Figs. 4 and 5). It is possible that these differences derive from different mechanisms of action of the two antibiotics. X-537 A has been shown to promote catecholamine release from chromaffin granules by direct transport of the monoamines across the membrane rather than the secondary result of an increase in membrane Ca^{2+} permeability induced by the ionophore [7–10]. This conclusion was based on the fact that X-537 A activity was independent of external Ca^{2+} concentration and that A 23187 which equilibrates Ca^{2+} gradients across biological membranes did not induce catecholamine release. It is also supported by experiments dem-

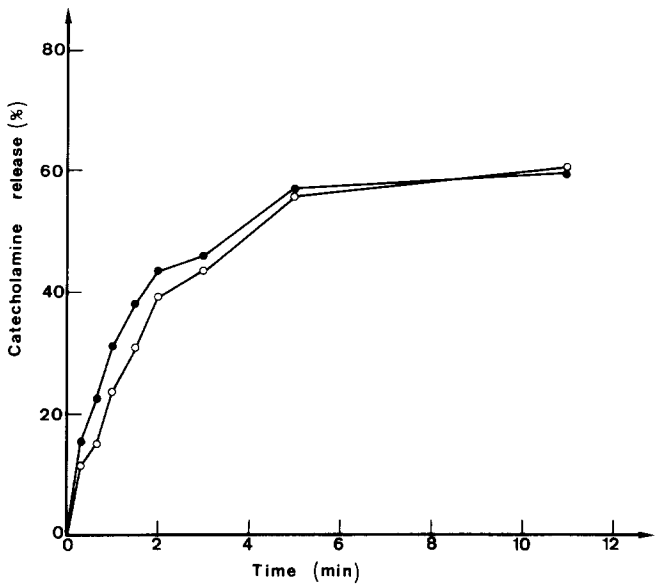

Fig. 5. Kinetics of adrenaline (●) and noradrenaline (○) release induced by X-14547 A. Chromaffin granules (0.16 mg protein) were incubated with X-14547 A (50 μg/mg protein) for various periods of time, in 10 mM Hepes (pH 7.0), 0.3 M sucrose (2 ml, final volume). They were then rapidly filtered on Millipore filters (HAWP) and the filtrates were assayed for adrenaline and noradrenaline. For each amine, data are expressed as percentage of controls lysed in 10 mM Hepes (pH 7.0). Hypoosmotic lysis released, per mg of initial granule protein: 1160 nmole adrenaline and 425 nmole noradrenaline. The release observed in absence of X-14547 A at $t = 0$ (less than 15% of the amounts released by hypoosmotic lysis) has been subtracted.

onstrating catecholamine transport across an artificial membrane by X-537 A in the absence of Ca^{2+} [21, 23]. Similar experiments with X-14547 A have not been done and we only know from the present work that this ionophore does not transport catecholamines across a chloroform phase (Table 1). This negative result could indicate that diffusion in a chloroform solution is not a good model system of transport across a biological membrane for X-14547 A. It should be noted that complexes of the two ionophores with primary amines have been shown to have different structures [4, 6] and they could exhibit different behaviour in the organic phase diffusion experiment described. But if the experiments described in Table 1 are relevant to the properties of biological membranes, then the negative result would imply that X-14547 A is not able to translocate directly monoamines and that this compound acts by a mechanism different from that of X-537 A. Different possibilities are currently under investigation.

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Table 1. Ionophore-mediated transport of noradrenaline across a chloroform phase*

Time (min)	Noradrenaline in the receiving compartment (μM)	
	with X 537 A	with X 14547 A
35	8	n.d. †
75	23	n.d. †
105	39	n.d. †
135	52	n.d. †
165	60	n.d. †

* pH of both aqueous phases was 7.0.
† n.d., not detected (less than 1 μM).

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