Preparation and Characterization of Reconstructed Small Intestinal Brush Border Membranes for Surface Plasmon Resonance Analysis

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Purpose. To prepare the surface generated by small intestinal brush border membrane vesicles (BBMVs) for the surface plasmon resonance (SPR) analysis, which allows the real-time measurement of binding events occurring on the intestinal membrane.

Methods. BBMVs were isolated from Sprague–Dawley rats, suspended in HEPES-buffered saline, and flowed over the surface of a SPR sensor chip composed of dextran derivatives modified with lipophilic residues. The surface coverage was determined from binding of bovine serum albumin to BBMV-immobilized sensor chip. The performance of BBMVs immobilized was evaluated by their interaction with otilonium bromide and bile salts.

Results. The stable BBMV surface was achieved when BBMV suspension was flowed over the sensor chip for 8 h at a rate of 2 μl/min. The flow of otilonium bromide resulted in an increased SPR signal because of its binding to calcium channel, which is known to be distributed over the gastrointestinal tact. When bile salts were flowed over ileal and duodenal BBMV surfaces, respectively, a slightly higher SPR signal was observed in the ileal BBMV surface, indicating the specific interaction of bile salts with bile acid transporters.

Conclusions. BBMV surfaces may be useful for the estimation of binding events on the intestinal membrane by SPR analysis, especially for the drugs that are orally administrated.

KEY WORDS: surface plasmon resonance; intestinal brush border membrane; otilonium bromide; bile salts.

INTRODUCTION

The surface plasmon resonance (SPR) biosensor, based on the detection of a refractive index change on a gold surface, has been widely used to investigate binding events occurring on biological surfaces (1,2). Without the need to label the ligands and complex sample preparation, SPR rapidly offers valuable information on the rate and extent of adsorption, association/dissociation kinetics, and the affinity constants of specific ligand–receptor interactions (3,4). In addition, SPR is one of the few applicable techniques that allows monitoring dynamic interactions within a fluid environment that is similar to conditions encountered *in vivo*. Therefore, for the SPR analysis many researchers have attempted to create biomimetic membrane surfaces composed of phospholipids and receptor proteins suitable for binding of ligand molecules (1,5–7).

On passive and/or active transport, most drugs developed for oral administration are absorbed in the gastrointestinal tract followed by systemic circulation. There are a lot of *in vivo* and *in vitro* methods that can be used to investigate the transport mechanism of drugs. Because *in vivo* investigations are, however, time-consuming and often difficult to quantify the results, it is evident that the predictive *in vitro* model is needed. To date, passive transcellular diffusion of the drug has been estimated *in vitro* by using Caco-2 cells (8), lecithin vesicles (9), and liposome surfaces (10). It is of importance to note that although the quantitative measurement of the passive transport is readily predicted using above methods, estimation of the active transport has been hindered by the need of cells expressing a sufficient amount of carrier proteins. Therefore, small intestinal brush border membrane vesicles (BBMVs), prepared from the small intestinal mucosa, are gaining recognition as an alternative tool to investigate the active transport because they contain phospholipids, hydrolytic enzymes, and carrier proteins that are responsible for many biologic binding events (11,12). In particular, BBMVs can be used to estimate the absorption of drugs and nutrients that may be orally administrated because of their structural and functional similarity to real intestinal membrane (13,14). Nevertheless, the extended use of BBMVs has often been hampered by the need of chromophoric or radiolabeled compounds.

In a present study, we describe a novel method to estimate binding events occurring on intestinal membranes, based on the SPR technology. In an attempt to mimic the real intestinal surface capable of interacting physically and/or actively with certain biological molecules, we attached BBMVs, isolated from Sprague–Dawley (SD) rats, on the surface of the sensor chip composed of dextran matrix modified with lipophilic residues (Fig. 1). The performance of the BBMV surface was examined by flowing the calcium channel blocker (otilonium bromide) and bile salts (cholate, taurocholate, deoxycholate; CA, TCA, DOCA, respectively) over the sensor chip for SPR analysis because the intestinal membrane is known to possess a considerable number of calcium channels and bile acid transporters.

MATERIALS AND METHODS

Materials

Bovine serum albumin (BSA), CA, TCA, and DOCA were purchased for the SPR experiment from Sigma (St. Louis, MO, USA). Otilonium bromide was kindly donated by DAE HWA Pharm. Co., Ltd., Korea. The SPR instrument was a Biacore 3000 (Biacore AB, Uppsala, Sweden), equipped with a L1 sensor chip. The L1 chip consisted of a carboxymethyldextran hydrogel derivatized with lipophilic alkyl chain anchors on a gold film and contained four channels with dimension of 2.4 × 0.5 × 0.05 mm (l × w × h), thereby providing a probing spot for the SPR signal of 0.26 mm^2 for each channel. All SPR experiments were conducted under a

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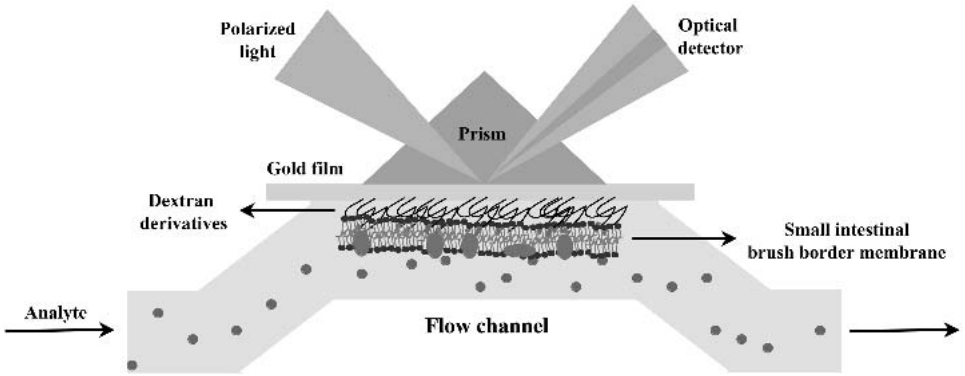

Fig. 1. Schematic illustration of the SPR experiment. Analyte solution is passed over the sensor chip covered with small intestinal brush border membrane vesicles (BBMVs). The SPR sensorgram is generated by the change in refractive index that occurs because of the binding of an analyte to the BBMV surface.

constant flow rate of 2 μl/min in running buffer, which was HEPES-buffered saline (10 mM HEPES, 150 mM NaCl, pH 7.4).

BBMV Preparation

For the preparation of BBMV, duodenum and ileum were obtained from the small intestines of SD rats (200–220 g fasted overnight; Refs. 14,15). Briefly, a homogenate of the mucosa scraped with a glass slide was prepared in buffer A (2 mM Tris-HCl, 50 mM mannitol, pH 7.1). Thereafter, $CaCl_2$ was added to give a concentration of 10 mM, allowed to stand at 4°C for 20 min, and centrifuged at 3000g for 15 min. The supernatant was then centrifuged again at 27,000g for 30 min. The pellet obtained was washed twice with buffer B (10mM Tris-HCl buffer, 300 mM mannitol, pH 7.1).

BBMV Characterization

The size distribution of BBMVs were observed using the dynamic light scattering (DLS, BI-9000AT, New York, NY, USA). DLS measurements were conducted using the helium ion laser system (Spectra Physics Laser Model 127-35, San Francisco, CA, USA) which was operated at 633 nm and 25 ± 0.1°C. The purity of BBMV was determined by established procedure for the activity assay of alkaline phosphatase (16) and Na^+K^+ ATPase (17). The binding study of otilonium bromide was performed by using ileal BBMVs whereas that of bile salts was performed by using both duodenal and ileal BBMVs (see below for detailed SPR experiments).

BBMV Immobilization on the Sensor Chip

Before its use, the surface of the L1 chip was conditioned with a 2.5-min injection of 20 mM 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS, Sigma) at a flow rate of 2 μl/min, followed by washing with a running buffer. The BBMV suspension with the concentration of 0.9 mg protein/ml was immediately injected until a response unit (RU) value reached 6000–8000 and the running buffer was continuously flowed to remove any multilamellar structures, resulting in no significant decrease in the response level (< 0.2 RU/min). The surface coverage was determined from BSA binding to the BBMV-immobilized sensor chip because it is known that BSA interacts strongly with dextran matrix on the sensor chip but does not with phospholipid-based layers (18).

SPR Experiments

Substances for the SPR analysis such as otilonum bromide and bile salts were dissolved in HEPES-buffered saline at predetermined concentrations. Each solution was injected for 5 min into the BBMV layer reconstructed on the L1 chip, and the solution was replaced with the running buffer to induce the dissociation of the complex. All the sensor surfaces were readily regenerated by a 3-min injection of 100 mM HCl between sample injections. The complete removal of the BBMVs from the L1 chip was achieved by an injection of 20 mM CHAPS. The BIAevaluation software (version 3.1) was used for the analysis of a sensorgram.

RESULTS AND DISCUSSION

Formation of the Reconstructed BBMVs

The size distribution and mean diameter of BBMVs prepared in this study were 169–339 nm and 245 nm, respectively, as demonstrated by DLS measurements (Fig. 2). For the formation of the real intestinal membrane on the SPR sensor chip, these BBMVs that were obtained from SD rats were

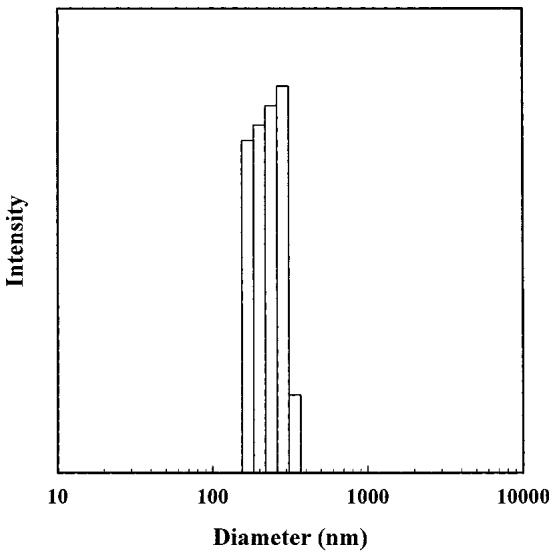

Fig. 2. Size distribution of BBMVs in HEPES-buffered saline measured by the dynamic light scattering at 25°C.

passed over the surface of the L1 chip containing dextran derivatives modified with lipophilic residues. A typical SPR sensorgram for the BBMV immobilization on the L1 chip is shown in Fig. 3. Because BBMVs were not covalently attached to the L1 chip but were immobilized by physical interactions, it was of paramount importance to find conditions capable of retaining BBMV without a significant loss. The surface saturation of BBMVs was achieved within 30 min when the protein concentration in the BBMV suspension was at least higher than 0.7 mg/ml. It was also found that a flow rate should be less than 5 μl/min to retain BBMV onto the surface; otherwise, the response level did not reach a steady state due to the significant drift of loosely bound BBMVs. After the injection of the BBMV suspension, the response level decreased rapidly at a rate of 65 RU/min for 5 min, followed by a slower decrease at 0.7 RU/min for 8 h (Fig. 3). In an additional duration of time, the response level was stabilized with a drift < 0.2 RU/min, resulting in a signal of ca. 6000–8000 RU; for example, it can be observed in Fig. 3 that, after the injection of the BBMV suspension, the SPR signal was stabilized at an almost constant level of 6000 RU. The deviation of stable response level might appear due to the flexability and roughness of the dextran matrix (ca. 100 nm in thickness) that possesses a larger surface area than a flat surface (19). To assess the surface coverage of the sensor chip, BSA (0.1 mg/ml) responsible for nonspecific binding to the dextran matrix was passed immediately after BBMV immobilization. BSA injection to the BBMV-immobilized sensor chip resulted in a slight increase in the SPR signal (< approx. 50 RU), whereas a significant increase over approx. 500 RU was detected for the dextran matrix in the absence of BBMVs. This indicates that the dextran matrix is no longer accessible by the BBMV-rich sensor surface.

For the repeated use of BBMV surface for SPR analysis, we attempted to find the condition by which reconstruction of the BBMV surface is achieved throughout the dissociation of bound analytes without a significant deterioration. It should be noted that the regeneration of the BBMV surface by 100 mM HCl was not achieved until a RU value reached the stable level, which took at least 8 h. However, once stabilized on the sensor chip, BBMVs were readily regenerated without a significant loss of the surface coverage by a 3-min treatment of 100 mM HCl between sample injections; the amount of BBMVs bound to the sensor chip was maintained for at least 10 cycles. This stability for repeated use was comparable with the results obtained by Cooper *et al.* (6), who prepared the supported phospholipid monolayer on the hydrophobic sensor chip, consisting of an octadecane-thiol self assembled monolayer on a gold surface. When the BBMV surface was treated with 10–100 mM NaOH, one of typical solutions for the regeneration of the phospholipid layer, most BBMVs were washed out because of the disruption of the interaction between lipophilic moieties of dextran derivatives and BBMV constituents. It is believed that these unique conditions for BBMV immobilization on the L1 sensor chip are principally governed by BBMV composition containing phospholipids with different charges and proteins. Although BBMV composition is a little bit different depending on preparation methods and animal species, a significant portion (> 40%) is known to be occupied by proteins (14,20). Because the hydrophobic interaction between phospholipids and lipophilic moieties of dextran matrix is the most important factor for a capture on the sensor chip, the stable immobilization of BBMVs might be limited within the specific condition, found in this study, primarily due to the lack of phospholipids in BBMVs. In a recent study, Okumura *et al.* prepared BBMVs which were isolated from midguts of the diamondback moth *Plutella xylostella* to examine their binding interactions to Cry1Ac, an insecticidal protein of *Bacillus thuringiensis*, by using SPR (21). They demonstrated that BBMV alone could not be immobilized on a flat hydrophobic sensor chip due to the low value of the phospholipid ratio against the BBMV protein, whereas the mixture of 95% phospholipid and 5% BBMV allowed stable reconstruction by the conventional regeneration procedure. In contrast to the L1 sensor chip which opens the possibility to work with phospholipid bilayers, the flat hydrophobic sensor chip composed of an octadecane-thiol self assembled monolayer on a gold surface allows formation of a flat rigid monolayer upon immobilization of phospholipid vesicles (6). Because BBMVs should be immobilized onto the sensor chip with a bilayer form to retain their binding activity, similar to the intestinal membrane, a flat hydrophobic chip may be not suitable for the immobilization of BBMV. The stable immobilization of BBMVs on the L1 sensor chip is believed to be achieved by the larger surface area than a flat surface, due to the roughness of the hydrogel surface. Another possible reason for the successful BBMV immobilization might be due to the flexible hydrogel barrier composed of dextran derivatives with a thickness of 100 nm, which increases the probability of the hydrophobic interaction (see Fig. 1).

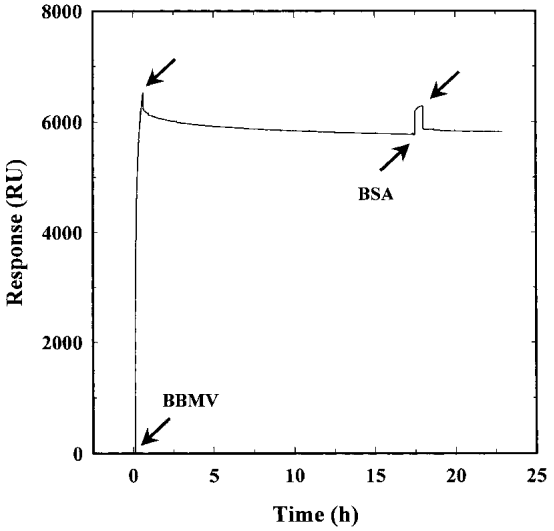

Fig. 3. Formation of BBMV surface on the sensor chip. The BBMV suspension was injected at a flow rate of 2 μl/min over the sensor chip, which had been conditioned with CHAPS, until a response unit value reached 6000–8000. Excess BBMVs were removed by washing with a HEPES-buffered saline for at least 8 h, followed by a 3-min injection of BSA (0.1 mg/ml) at a rate of 2 μl/min; once stabilized, free BBMV surface was readily regenerated by a 3-min treatment of 100 mM HCl. The resulting BBMV surface was very stable with a drift < 0.2 RU/min. Arrows represent the beginning and end of each injection.

Otilonium Bromide Binding to BBMV

The performance of BBMVs attached on the L1 chip was estimated by flowing different concentrations of otilonium

bromide at a rate of 2 μl/min (Fig. 4). Upon the injection of otilonium bromide, the SPR signal rapidly increased by the association with BBMVs, followed by a slower increase to reach equilibrium. After the end of injection, the replaced running buffer dissociated bound otilonium bromide. The SPR signal was well correlated with the initial otilonium bromide concentration, indicating that the amount of otilonium bromide bound to BBMV was dependent on the concentrations.

Otilonium bromide, a quaternary ammonium derivative, has been used as a spasmolytic agent for the treatment of intestinal disorders, particularly for the irritable bowel syndrome (22). Studies have shown that its spasmolytic action is mainly attributed to its interfering effect on Ca^{2+} mobilization from both intra- and extra-cellular sources (23,24). Thus, owing to its role as a calcium channel blocker, the injected amount of otilonium bromide might correlate with the SPR signal, as shown in Fig. 4. However, it should be emphasized that although the calcium channel primarily accounts for the binding affinity of otilonium bromide to BBMVs, other receptors that are distributed over the gastrointestinal tract may partially contribute to enhanced binding, for example, several muscarinic and tachykinin NK2 receptors are known to possess sub μM affinity to otilonium bromide (24,25).

Bile Acids Binding to BBMV

Bile acids, essential for the digestion and absorption of lipid and lipid-soluble vitamin, are amphiphilic molecules synthesized from cholesterol in the liver. They are stored in the gall bladder, secreted into the small intestine, and subsequently reabsorbed by both passive and active transport. This interesting feature to preserve the total amount of bile acids in the body (e.g., 2.5–5 g in human) is called the enterohepatic circulation, which is mediated by the high efficacy of both intestinal and liver absorptions (26). For the intestinal absorption of bile acids, ileum plays an important role because it possesses bile acid transporter that deliver bile acids to the liver via the portal vein. This active transport system has drawn a great attention for the use of bile acids as a potential drug carrier to augment the oral absorption and bioavailability of drugs (26–28).

Although many researchers have studied the intestinal and hepatic absorption of bile salts, the literature has not focused on the real-time interaction along the small intestine. In this study, we attempted to observe detailed binding profiles of bile salts to the intestinal membrane by using a SPR instrument. CA and its taurine conjugate (TCA) were chosen for the SPR analysis because they are main primary bile salts.

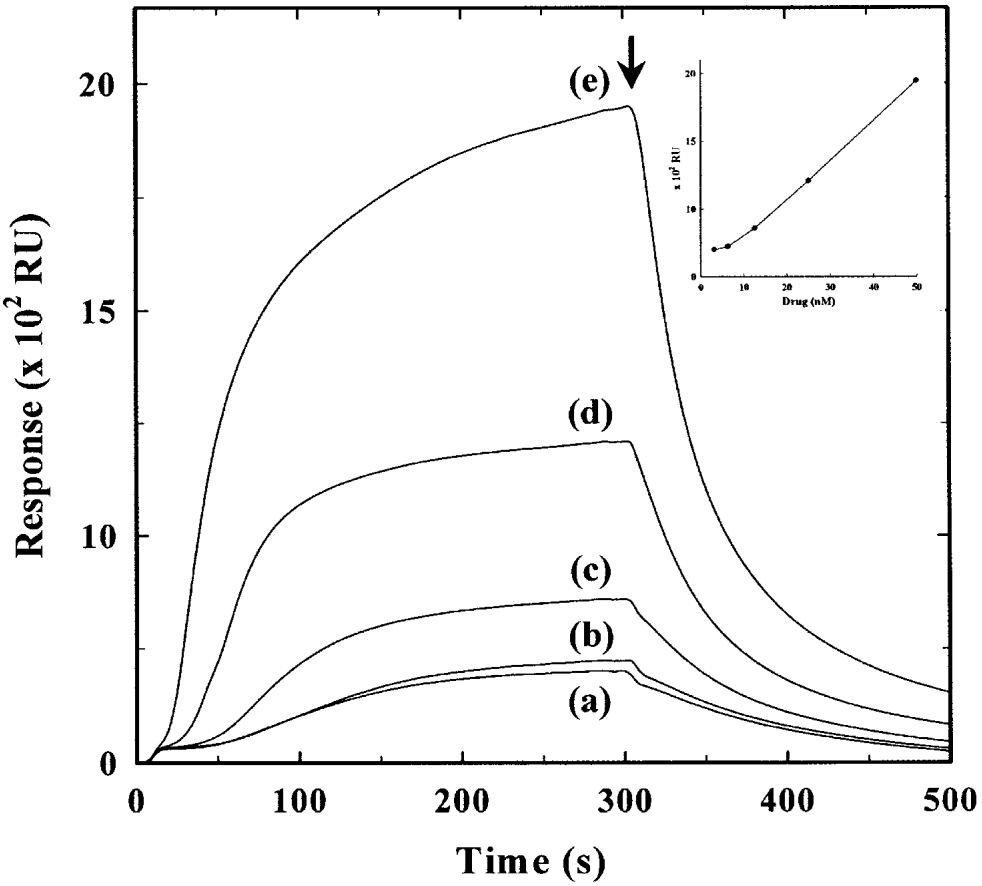

Fig. 4. SPR response for binding of otilonium bromide to BBMV surface. Five different concentrations of otilonium bromide were injected over the BBMV bound to the sensor chip at a flow rate of 2 μl/min for 5 min in the HEPES-buffered saline: (a) 3.12 nM; (b) 6.25 nM; (c) 12.5 nM; (d) 25 nM; (e) 50 nM. The arrow indicates the end of injection (t = 300s). The responses at t = 300s vs. the concentration of otilonium bromide were inserted.

DOCA, produced by the action of the intestinal bacteria, was also tested as a representative secondary bile salt (26,27). The binding events were observed from the surfaces of BBMVs, in which ileal and duodenal membranes were separately loaded onto different sensor channels. The results demonstrated that SPR signals of all the bile salts rapidly increased in both ileal and duodenal BBMV surfaces due to the presence of bound bile salts, which were then rapidly dissociated when the solution was replaced with the running buffer (Fig. 5). The rapid dissociation might occur due to the amphiphilic nature of bile salts and shear stress generated by a flow of the running buffer. For both BBMV surfaces, the maximum RU values were in the order of DOCA > TCA > CA; these different binding behaviors may partially contribute to the absorption rates of bile salts, demonstrated by other groups (28–30).

The SPR signals for bile salts in duodenal BBMVs might have resulted from non-specific physical interactions because receptors for specific interactions with bile salts are known to be absent in duodenum. It is of importance to note that, when compared to duodenal BBMV, each of bile salts revealed a slightly higher SPR signal in ileal BBMV during injection time. This result obviously suggests the specific interaction of bile salts with bile acid transporters in ileal BBMV. Along this line of research, the detailed binding affinity and kinetic for different bile salts and macromolecular drugs for oral administration are currently under investigation.

CONCLUSION

We prepared the BBMV-immobilized sensor chip for the SPR analysis. The stable BBMV surfaces could be used repeatedly by the regeneration with 100 mM HCl and they showed reasonable binding kinetics for otilonium bromide and bile salts. Based on these results, it was evident that for the characterization of binding events occurring on intestinal surface, this *in vitro* system exhibited advantages such as the lack of labeling requirements, the real-time acquirement of various results, and the repeated use for various compounds.

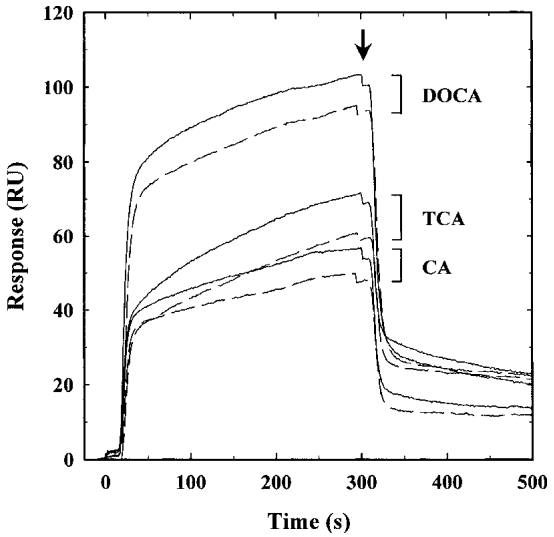

Fig. 5. SPR response for binding of bile acids to BBMV surface. Ileal (solid line) and duodenal (dashed line) BBMVs, obtained from SD rats, were separately attached to the sensor chip, after which CA, TCA, and DOCA (100 μM) were injected at a flow rate of 2 μl/min for 5 min in the HEPES-buffered saline. The arrow indicates the end of injection (t = 300 s).

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