Potentiation by Indomethacin of Receptor-Mediated Catecholamine Secretion in Rat Adrenal Medulla

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Received September 2, 1996 Accepted December 9, 1996

ABSTRACT—Effects of indomethacin on catecholamine secretion evoked by receptor agonists, muscarine, bradykinin or histamine, in rat adrenal chromaffin cells were studied. Indomethacin at 200 μM increased a sustained component of secretion during stimulation with muscarine, bradykinin and histamine by a factor of 2.3, 2.1 and 2.9, respectively, whereas it did not significantly alter basal, high-K+ and nicotine-evoked secretions. Although indomethacin at above 400 μM dose-dependently increased basal secretion, the amount of secretion induced by indomethacin alone was much smaller than that in muscarine-evoked secretion as compared at the same concentration of indomethacin applied. Bradykinin-evoked secretion and its potentiation by indomethacin were not inhibited by 20 μM nifedipine but were suppressed by 0.5 mM Ni2+. The cyclooxygenase inhibitor, ibuprofen (200 μM) did not mimic the effect of indomethacin; prostaglandin E2 (20 μM) and arachidonic acid (100 μM) did not significantly alter either bradykinin-evoked secretion itself or its potentiation by indomethacin. Bradykinin increased the intracellular free Ca2+ concentration, [Ca2+]i, in cells loaded with indo-1, and this response was enhanced in the presence of indomethacin. These results suggest that indomethacin may promote Ca2+ entry to potentiate agonist-evoked catecholamine secretions through a novel action that is not directly related to the inhibition of cyclooxygenase activity with indomethacin.

Keywords: Adrenal chromaffin cell (rat), Catecholamine secretion, Indomethacin, Muscarine, Bradykinin

Nicotinic cholinergic transmission from the splanchnic nerves plays a central role in catecholamine (CA) secretion from adrenal medullary cells. However, the stimulation of non-nicotinic receptor agonists such as muscarine, bradykinin and histamine also evokes CA secretion in the perfused rat adrenal gland (1–6). These agonists promote phosphoinositide metabolism in chromaffin cells (7, 8). In many cell types, agonist-induced phosphoinositide metabolism is accompanied by the activation of phospholipase A2 to release arachidonic acid (9). Thus, there occurs the possibility that arachidonic acid metabolism may participate in the regulation of secretory processes in chromaffin cells. Although this possibility has been tested by various investigators, reported effects of phospholipase A2 inhibitors (10, 11), arachidonic acid (12–15), cyclooxygenase inhibitors (16, 17) and prostaglandins (17–21) on secretory responses have not yet provided a clear view on the role of arachidonic metabolism in CA secretion in rat chromaffin cells. During the course of the study, I found that indomethacin markedly potentiates receptor-mediated CA secretions. Thus, this indomethacin effect was studied in detail to determine if it derived from the inhibition of cyclooxygenase activity by this drug. Results obtained suggest that indomethacin enhances Ca2+ entry in agonist-stimulated chromaffin cells by a mechanism that is not directly associated with the inhibition of cyclooxygenase. This novel action of indomethacin may be useful for investigating the mechanism of Ca2+ entry during receptor stimulation in chromaffin cells and other cell types.

A preliminary report of this study has appeared in abstract form (22).

MATERIALS AND METHODS

Chemicals and solutions
Muscarnine, prostaglandin E2 (PGE2), arachidonic acid and nifedipine were purchased from Sigma Chemical (St. Louis, MO, USA). Indo-1 AM, fura-2 AM and HEPES
were from Dojin Laboratory (Kumamoto). Pluronic F-127 was from Behring Diagnostics (La Jolla, CA, USA). Bradykinin was from Peptide Institute (Minoh). Calcium Green-1 AM was from Molecular Probes (Eugene, OR, USA). Histamine, indomethacin, ibuprofen, neostigmine and inorganic chemicals were from Wako Pure Chemical (Osaka). The standard medium to perfuse isolated rat adrenal glands was a modified Krebs solution that consisted of 150 mM NaCl, 5.6 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose and 1 mM NaH₂PO₄·10 mM HEPES buffered at pH 7.4 by titration with NaOH. Concentrated solutions of indomethacin, ibuprofen, PGE₂, arachidonic acid and nifedipine were prepared in dimethyl sulfoxide and diluted in the standard medium. The final concentration of dimethyl sulfoxide was either 0.1% or 0.2%, which did not affect secretory or intracellular free calcium concentration ([Ca²⁺]ᵢ) responses evoked by the receptor agonists.

**Monitoring of CA secretion**

CA secretion from the isolated rat adrenal gland was continuously detected by the previously described method (3). In brief, adrenal glands were isolated from male Wistar rats (340 - 430 g) anesthetized by i.p. injection of pentobarbital (50 mg/kg). The gland was perfused retrogradely at a rate of 0.15 ml/min with the standard medium through a cannula inserted into the adrenal vein. The perfusate was then let into a flow cell (TL-5; Bioanalytical Systems, West Lafayette, IN, USA) in which glassy carbon electrodes were installed. The amount of CA in the perfusate was then let into a flow cell (TL-5) with an amperometric detector (VMD-101A; Yanaco, Kyoto) at an applied electrode voltage of 0.4 V. The intensity of oxidation current that is proportional to the amount of CA in the perfusate was measured with an amperometric detector (VMD-101A; Yanaco, Kyoto) at an applied electrode voltage of 0.4 V. The intensity of oxidation current that is proportional to the amount of CA in the perfusate was subjected to 12-bit A/D conversion and recorded on a magnetic disk. Figures presented in this paper were drawn by an X-Y recorder with data saved on the magnetic disk. Experiments were carried out at room temperatures (24 - 27°C).

For quantitative evaluations, response sizes were calculated numerically using stored data and were then converted into moles of CA based on a calibration relation obtained with adrenaline standard solution.

Statistical differences between means of CA secreted during determined periods were calculated by Student's t-test for non-paired data. Level of significance was established at the value of P<0.05.

**Measurement of changes in [Ca²⁺]ᵢ**

Changes in [Ca²⁺]ᵢ arising in chromaffin cells in the perfused rat adrenal medulla were measured as described in a previous paper (23). The adrenal gland was perfused for 60 min recurrently with 2 ml of the standard medium containing indo-1 AM at 10 μM, 0.1% of Pluronic F-127 and 20 μM neostigmine to load chromaffin cells with the indicator dye. About half of the adrenal cortex was then removed by dissection and the exposed portion of adrenal medulla under the perfused condition was then subjected to 12-bit A/D conversion and recorded on a magnetic disk with a system similar to that used for secretion experiments.

**RESULTS**

**Effects of indomethacin on muscarine-evoked CA secretion**

A continuous 18-min stimulation of a perfused adrenal gland of the rat with 100 μM muscarine evoked a biphasic secretory response, an initial transient followed by a sustained secretion as shown in Fig. 1Aa. The presence of 200 and 800 μM indomethacin in the perfusate from the 6th to 12th min during the 18-min stimulation with muscarine markedly potentiated the sustained secretion as shown in Fig. 1Ab. Namely, the response in the period during which indomethacin was applied (between the 6th and 12th min) was separated into the two parts, area A and area S, by the line L. The increase of muscarine-evoked CA secretion by indomethacin was evaluated as illustrated in Fig. 1Ab. Namely, the response in the period during which indomethacin was applied (between the 6th and 12th min) was separated into the two parts, area A and area S, by the line L. The indomethacin effect is expressed either by the amount of increased secretion calculated from the area A or by the fractional increase of secretion, (1 + A/S). The slope of line L, being -1.6±0.9% per min (mean±S.E., n=5), was obtained from control data such as that shown in Fig. 1Aa.

The indomethacin-induced increase of muscarine-evoked CA secretion was calculated from the area A and is plotted, as a function of indomethacin concentration, by the filled circles in Fig. 1B. Marked increases of the secretion occurred with indomethacin at concentrations above 100 μM. The increase seems to level off at indomethacin concentrations higher than 1.6 mM, although no such experiments were carried out because of the limitation of indomethacin solubility.

The solid line in Fig. 1B was drawn based on the Hill equation:
equation with the coefficient of 1.9, which was determined by a least-squares fitting of the data points obtained with indomethacin at concentrations above 100 μM. This analysis indicates that the dose of indomethacin for the half-maximal effect is 460 μM, where the fractional increase of the muscarine-evoked secretion is close to 5.

The rate of basal CA secretion in the perfused rat adrenal gland at room temperatures was lower than 5 pmol/min (23). Indomethacin at concentrations below 200 μM had a negligible effect on the basal level as shown in Fig. 2a. However, indomethacin alone induced CA secretion in a dose-dependent manner at concentrations above 400 μM; the open circles in Fig. 1B show the amount of CA secreted during a 6-min application of indomethacin at concentrations indicated by the abscissa. The indomethacin-induced secretion above the basal level was much smaller than the indomethacin-induced increase in muscarine-evoked secretion as shown in Fig. 1B.

The effect of indomethacin on CA secretion evoked by various stimulants

In rat adrenal chromaffin cells, receptor agonists such as muscarine, bradykinin and histamine evoke CA secre-
tion, of which characteristics are similar in many aspects (3–5). When 200 nM indomethacin was administered between the 6th and 12th min during a 18-min stimulation with 200 nM bradykinin (BK). c: Indomethacin was present from 5 min prior to the 10-min stimulation with 200 nM bradykinin to the end of the measurement. d, e and f: Indomethacin was applied between the 6th and 12th min during a 18-min stimulation with 100 μM histamine (His), 20 mM extracellular K⁺ and 10 μM nicotine (Nic), respectively. The vertical and horizontal scales at panel a apply to all of the traces in this figure. Two or three tests with different secretagogues were carried out with one gland. Each trace is representative of four consistent results.

Fig. 2. Effects of 200 μM indomethacin on CA secretions evoked by various secretagogues. a: Influence of indomethacin (indom.) on the basal secretion. b: Indomethacin was applied between the 6th and 12th min during a 18-min stimulation with 200 nM bradykinin (BK). c: Indomethacin was present from 5 min prior to the 10-min stimulation with 200 nM bradykinin to the end of the measurement. d, e and f: Indomethacin was applied between the 6th and 12th min during a 18-min stimulation with 100 μM histamine (His), 20 mM extracellular K⁺ and 10 μM nicotine (Nic), respectively. The vertical and horizontal scales at panel a apply to all of the traces in this figure. Two or three tests with different secretagogues were carried out with one gland. Each trace is representative of four consistent results.

Effects of various agents on bradykinin-evoked CA secretion and its potentiation by indomethacin

Indomethacin is well known as a cyclooxygenase inhibitor. Thus, whether the observed effects of indomethacin on secretory responses were related to cyclooxygenase metabolism or not was assessed. At first, the
effect of ibuprofen, another cyclooxygenase inhibitor, was tested in the period between the 6th and 12th min during a 18-min stimulation with bradykinin as shown in Fig. 3a. Unlike indomethacin, 200 nM ibuprofen did not promote the bradykinin-evoked secretion. Ibuprofen at 400 nM was also ineffective for promoting bradykinin-evoked secretion (data not shown).

In the experiment shown in Fig. 3b, the effect of 20 nM PGE2 on indomethacin-induced increase in bradykinin-evoked secretion was studied. The application of PGE2, commenced at 5 min before the bradykinin stimulation (indicated by the arrowhead), produced a small secretory response. The continued presence of PGE2, however, did not affect either bradykinin-evoked secretion or its increase by indomethacin. The fractional increase of the bradykinin-evoked secretion due to 200 nM indomethacin in the presence of PGE2 is 2.3±0.1 (n=4), which does not significantly differ from the corresponding value in the absence of the exogenous PGE2 (P>0.05, t-test).

Moreover, 100 nM arachidonic acid applied during stimulation with bradykinin had no effect (Fig. 3c).

Effects of ibuprofen (200 nM), PGE2 (20 nM) and arachidonic acid (100 nM) on muscarine-evoked secretion were examined with the same protocol as in the experiments with bradykinin. Although only a single experiment with each agent was performed, no essential difference was recognized as compared with the results obtained with bradykinin. Although the doses of the above agents to assess their effects were not chosen on rigorous bases, the concentrations used are considered to be high enough for observing their effects, if they exist. Thus, the above results suggest that the promoting effect of indomethacin on receptor-mediated CA secretions is not derived from the inhibition of cyclooxygenase activity but from a novel effect of the drug.

At least two pathways of Ca2+ entry are involved in the secretory responses of rat chromaffin cells. One involves voltage-gated Ca2+-channels that play an essential role
when cells respond to depolarizing stimulants such as nicotinic agents and excess extracellular K+. The other pathway is used for receptor-mediated Ca\(^{2+}\) entry that supports CA secretion evoked by agonists such as muscarine, bradykinin and histamine. In Fig. 3d, the effect of indomethacin on bradykinin-evoked CA secretion was examined in the presence of 20 \(\mu\)M nifedipine, which effectively blocks voltage-gated Ca\(^{2+}\) channels in chromaffin cells (3, 4). In the presence of nifedipine, bradykinin-evoked CA secretion was slightly reduced: 88±9% (n=4) of the control when evaluated by the amount of CA secreted during the initial 6-min stimulation. However, the fractional increase as the index of the indomethacin effect is calculated to be 1.9±0.1 (n=4), which does not significantly differ from that in the absence of nifedipine (\(P > 0.05\), t-test).

Receptor-mediated Ca\(^{2+}\)-entry in chromaffin cells is effectively blocked by Ni\(^{2+}\) (25). The extent of inhibition of bradykinin-evoked secretion by 0.5 mM Ni\(^{2+}\) depended on the phase of the secretory response, showing 18% inhibition in the initial phase of secretion, whereas 75% inhibition occurred in the sustained phase. As shown in Fig. 3e, the sustained secretion evoked by bradykinin as well as its increase by indomethacin were extensively suppressed.

The results obtained with nifedipine and Ni\(^{2+}\) are compatible with the idea that indomethacin potentiates agonist-evoked CA secretion by increasing receptor-

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**Fig. 4.** Effects of indomethacin on [Ca\(^{2+}\)]\(_{\text{c}}\) change induced by bradykinin and excess extracellular K\(^+\) in indo-1-loaded chromaffin cells of the perfused rat adrenal medulla. a: Indomethacin (indom., 200 \(\mu\)M) was applied between the 6th and 12th min during a 18-min stimulation with 200 nM bradykinin (BK). The top and middle traces respectively show changes in intensities of indo-1 fluorescence at 405 and 480 nm. The trace at the bottom indicates the ratio of intensities of 405 to 480 nm fluorescence. b: A set of the three traces obtained with the same experimental protocol as in panel a except that 20 mM K\(^+\), instead of bradykinin, was used as a secretagogue. The vertical scale, which applies to both traces at the bottom in panels a and b, indicates a 40% change in the fluorescence intensity ratio with respect to the resting levels. The records are representative of four consistent results from different glands.
mediated Ca\(^{2+}\) entry.

**The influences of indomethacin on agonist-induced changes in \([Ca^{2+}]\)**

In Fig. 4a, indo-1-loaded chromaffin cells were stimulated for 18 min by 200 nM bradykinin, during which 200 \(\mu\)M indomethacin was applied from the 6th to 12th min. The intensity of indo-1 fluorescence emitted at either 405 or 480 nm decreased during application of indomethacin most likely due to absorption of 350 nm (excitation wavelength for indo-1) light with indomethacin that was accumulated in the chromaffin cells (top and middle traces in Fig. 4a). However, the 405/480 nm ratio of indo-1 fluorescence revealed that indomethacin further increased \([Ca^{2+}]\) beyond the level of the bradykinin-induced sustained elevation of \([Ca^{2+}]\), (bottom trace in Fig. 4a).

The influence of indomethacin on \([Ca^{2+}]\), change induced by 20 mM K\(^+\) was examined as shown in Fig. 4b. The increase in \([Ca^{2+}]\) during the application of indomethacin was not significant.

Although the results are not shown, the indomethacin effect was also examined using fura-2 and Calcium Green. The ratio of fura-2 fluorescence excited at 342 nm to that at 380 nm, which normally represents the \([Ca^{2+}]\), level in fura-2 fluorometry, was either unaltered or decreased during application of indomethacin. This may result from modification of the fura-2 fluorescence ratio in a misleading manner since indomethacin absorbs more light at 342 nm than at 380 nm. When Calcium Green-1 was employed, the fluorescence intensity changed in a manner similar to that shown with indo-1 (Fig. 4a). Because both the excitation and emission wavelengths (470 and 530 nm, respectively) of Calcium Green are apart from the absorption bands of indomethacin, the result with Calcium Green-1 is reliable. Considering these results all together, the data with fura-2 were discarded.

**DISCUSSION**

**Potentiation by indomethacin of agonist-evoked CA secretions**

It was found in this study that indomethacin greatly potentiated muscarine-, bradykinin- or histamine-evoked CA secretion in the rat adrenal medulla (Fig. 1). In contrast, indomethacin increased high-K\(^+\) - or nicotine-evoked CA secretion only to the extent comparable with the amount of secretion induced by indomethacin alone. The use of indomethacin at concentrations between 25 and 200 \(\mu\)M is of particular interest since it significantly increases receptor-mediated secretions, whereas indomethacin at such concentrations has no substantial effect on either basal secretion or secretions evoked by depolarizing agents. This may be potentially useful for distinguishing intracellular processes induced by various secretagogues in chromaffin cells.

**The mechanism by which indomethacin potentiates agonist-evoked CA secretion**

In cells loaded with indo-1, the elevated \([Ca^{2+}]\) due to bradykinin stimulation was further increased during application of indomethacin with a time course similar to that observed in secretion experiments (Fig. 4a). On the other hand, high-K\(^+\)-induced increase in \([Ca^{2+}]\), was not significantly altered by 200 \(\mu\)M indomethacin (Fig. 4b). These are compatible with the results of secretion experiments. Therefore, the potentiation of CA secretion seems to be directly correlated with the indomethacin-induced increase in \([Ca^{2+}]\). Although rat chromaffin cells possess intracellular Ca\(^+\) stores that release Ca\(^+\) following receptor stimulation, this Ca\(^+\) poorly contributes to CA secretion when the medium contains Ca\(^{2+}\) at normal concentrations (26). Thus, the indomethacin-induced increase in \([Ca^{2+}]\), is thought to be associated with an increased Ca\(^{2+}\) entry. This view is consistent with the fact that both the sustained component of bradykinin-evoked secretion and its increase by indomethacin were inhibited (Fig. 3e) by Ni\(^{2+}\) which inhibits receptor-mediated Ca\(^{2+}\) entry (25).

Nifedipine, which selectively inhibits voltage-gated Ca\(^{2+}\) channels in this preparation (3), only slightly affectindomethacin-mediated potentiation of bradykinin-evoked secretion (Fig. 3d).

These results suggest that the receptor-mediated Ca\(^{2+}\) entry may be promoted by indomethacin. This may explain why indomethacin is particularly effective for promoting receptor-mediated CA secretions. Namely, indomethacin which may weakly stimulate Ca\(^{2+}\) entry even in the absence of receptor agonists strongly activates the receptor-mediated Ca\(^{2+}\) entry when it cooperates with receptor stimulations. Indomethacin may exert its action from the cell interior because the time course of the fluorescence decrease which indicates the accumulation of indomethacin in the intracellular space (top and middle traces in Fig. 4a) is similar to that of the increase in CA secretion during indomethacin application (Fig. 2b).

Indomethacin is widely used as a cyclooxygenase inhibitor. Some investigators have tested this effect on secretory responses. Sasakawa et al. (16) reported that indomethacin did not affect carbamylcholine-evoked CA secretion in bovine chromaffin cells. Yamada et al. (17) found that pretreatment with indomethacin slightly reduced CA secretion from perfused dog adrenal glands stimulated by acetylcholine or nicotine. These results are not in accordance with the present result. However, there are some difficulties in making such a comparison because
the range of indomethacin concentration used in this study is higher than those in the above studies. Furthermore, chromafl’in cells derived from various animal species may not be alike; in bovine chromafl’in cells, for instance, receptor-mediated Ca2+ entry mechanism is known to be poorly developed (27, 28).

Arachidonic acid and PGE2 are the substrate and one of products in cyclooxygenase metabolism. Effects of these agents on secretory responses in chromafl’in cells have also been examined by various investigators. PGE2 induced CA secretion (17–20) and potentiated secretions evoked by various secretagogues (17, 18, 21). PGE2 also induced an increase in [Ca2+]i due to the promotion of Ca2+ entry (29). These PGE2 effects somewhat resemble those shown, in the present study, with indomethacin rather than those with PGE2. PGE2 applied to the present preparation did not affect either bradykinin-evoked secretion or its increase by indomethacin (Fig. 3b), although a very weak secretion was evoked by PGE2 alone as reported in previous papers (17–20).

Despite a number of previous studies (12–16), the role of arachidonic acid on secretory responses in chromafl’in cells has not been established until now. In the present study, bradykinin-evoked secretion was not affected by the presence of arachidonic acid (Fig. 3c).

Although it is difficult to discuss these scattered facts in a consistent way, these results as a whole argue against the idea that the indomethacin effect described here originates from either the reduced production of PGE2 or the accumulation of arachidonic acid due to the inhibition of cyclooxygenase activity by the drug. This is supported by the fact that another cyclooxygenase inhibitor, ibuprofen, did not mimic the indomethacin effect (Fig. 3a). As one possibility, indomethacin may act as an activator of Ca2+ channels involved in the receptor-mediated Ca2+ entry. If this is proven as a novel action of indomethacin in future studies, it may be utilized to identify a class of Ca2+ channels that are activated after receptor stimulation in chromafl’in cells and other cell types.

Acknowledgments
This study was supported in part by Grants-in-Aid (06670050 and 07308074) for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

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