An M2 Muscarinic Receptor Subtype Coupled to Both Adenylyl Cyclase and Phosphoinositide Turnover

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To investigate whether a particular receptor subtype can be coupled to multiple effector systems, recombinant M2 muscarinic receptors were expressed in cells lacking endogenous receptor. The muscarinic agonist carbachol both inhibited adenylyl cyclase and stimulated phosphoinositide hydrolysis. The stimulation of phosphoinositide hydrolysis was significantly less efficient and more dependent on receptor levels than the inhibition of adenylyl cyclase. Both responses were mediated by guanine nucleotide binding proteins, as evidenced by their inhibition by pertussis toxin; the more efficiently coupled adenylyl cyclase response was significantly more sensitive. Thus, individual subtypes of a given receptor are capable of regulating multiple effector pathways.

THE BIOLOGICAL SIGNALS OF MANY hormones and neurotransmitters are transduced into target cells by interaction of their receptors with guanine nucleotide binding (G) proteins, which regulate the activity of effector enzymes such as adenylyl cyclase (AC) (*1*) and phospholipase C (PLC) (*2, 3*). Because many receptors have two or more distinct subtypes and are known to interact with more than one effector, correlations between the activation of a specific receptor subtype and a specific effector have been implied (*4, 5*). Muscarinic acetylcholine receptors (mAChRs) are coupled via G proteins to multiple effector systems including AC, PLC, and cardiac potassium channels (*5*). Three mAChR subtypes have been distinguished pharmacologically (*6*) and four genetically (*7*). Different mAChR subtypes seem to be coupled to different effector systems (*8–10*). In phospholipid vesicles, reconstituted cerebral mAChRs can interact with the AC inhibitory G protein (G_i) and with G_0, a G protein of unknown function (*11*). However, unambiguous interpretation of studies that use tissues or cells as a source of receptor has been hampered by the existence of multiple receptor subtypes. To better address this question we have studied the effector coupling of a single recombinant M2 mAChR subtype stably expressed in cells that lack endogenous mAChRs.

Chinese hamster ovary (CHO) cells were stably transfected with a vector directing the expression of the porcine atrial M2 mAChR complementary DNA (*12*). A mouse dihydrofolate reductase (DHFR) gene served as a selectable marker to isolate cell populations expressing various mAChR levels, by their resistance to the DHFR inhibitor methotrexate (*13*). The antagonist [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) bound to intact transfected CHO cells (*14, 15*) with an apparent dissociation constant (K_D) of 75 p*M*, similar to our observations for cell homogenates (63 p*M*) (*12*) and comparable to that of the native porcine atrial mAChR (47 to 61 p*M*) (*16*). The K_D for QNB was similar in cells expressing up to 2.5×10^6 receptors per cell.

In heart and brain, there are at least two agonist affinity states of the mAChR, probably resulting from interaction with G proteins (*5, 16–18*). Indeed, guanosine triphosphate (GTP) and its nonhydrolyzable analogs, which uncouple G proteins from receptors (*1*), convert high affinity mAChRs to low affinity (*5, 16–18*). In transfected cell homogenates, 30% of the mAChRs bound the agonist carbachol with high affinity (Fig. 1A). Guanosine 5′-(3-*O*-thio)triphosphate (GTPγS) converted 70% of the high affinity sites to a low affinity. The agonist oxotremorine recognized 24% of the mAChRs with high affinity, and GTPγS converted 45% of this population to a low affinity (Fig. 1B). These results (Table 1) are similar to those observed for native porcine atrial M2 mAChRs (*16*). The effect of pertussis toxin (PTX) on agonist binding was also studied (Fig. 1). PTX catalyzes the adenosine diphosphate (ADP)–ribosylation of certain G proteins, abolishing their interaction with receptors, which in turn decreases the affinity of receptors for agonists (*1*). PTX treatment of transfected cells decreased the number of high affinity carbachol and oxotremorine binding sites to an extent comparable to the decrease caused by GTPγS (Fig. 1 and Table 1), confirming that the M2 mAChR interacted with endogenous G proteins of CHO cells. In addition, multiple agonist binding states are a property of a single mAChR subtype; the high affinity state results from G protein interaction.

To determine whether the interaction of the recombinant mAChR with G proteins resulted in coupling to biochemical responses, we investigated the effects of carbachol on two second messenger systems, AC and PLC. Experimental conditions (*10, 19–21*) were selected to optimize these responses. Carbachol-induced inhibition of AC was determined from intracellular adenosine 3′,5′-monophosphate (cAMP) levels after stimulation with forskolin (*21, 22*). To ensure that changes in cAMP levels would not be influenced by changes in cAMP phosphodiesterase activity (*21*), we used the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX). Half maximal inhibition (ED_{50}) of forskolin-induced cAMP accumulation occurred at 7.1×10^{-8}*M* carbachol, and up to 76% of the 16.5-fold induction was inhibited by 10^{-4}*M* carbachol (Fig. 2A). Similar results were obtained

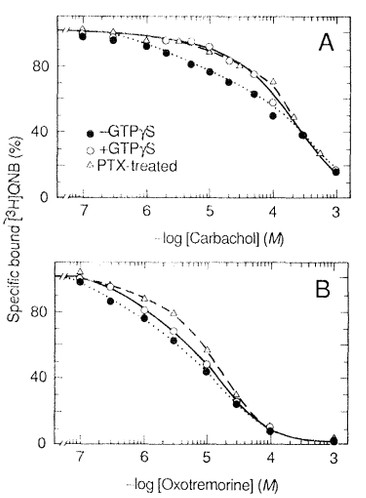

Fig. 1. Binding of muscarinic agonists and the effect of GTPγS and pertussis toxin (PTX). The displacement of [^{3}H]QNB binding by (**A**) carbachol and (**B**) oxotremorine was studied in homogenates from transfected CHO cells expressing 1.45×10^6 mAChRs per cell (*14*). The cells were incubated for 4.5 hours in the absence (● and ○) or presence (△) of PTX (100 ng/ml) prior to homogenization. Homogenates (15 μg of protein, 150 p*M* QNB sites) of untreated cells were assayed in the absence (●) or presence (○) of GTPγS (100 μ*M*). Homogenates of PTX-treated cells were assayed in the absence of GTPγS (△). The specific binding of [^{3}H]QNB was not affected by the various treatments and ranged from 64.8 to 65.9 p*M*. Each value is the mean of at least three experiments. Standard errors were normally less than 10% of the means. Computer-fitted binding parameters are summarized in Table 1.

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with incubation times of 10 or 30 minutes. Treatment of the transfected CHO cells with carbachol also resulted in enhanced formation of inositol phosphates. Carbachol evoked a 3.5- to 4.8-fold increase in the formation of inositol trisphosphate (IP_3) and bisphosphate (IP_2), the primary products of PLC-catalyzed hydrolysis of polyphosphoinositides (*2*). The increase in IP_3 and IP_2 formation occurred rapidly, reaching a maximum within 1 minute; this was followed by a slower linear accumulation of inositol monophosphate (IP_1), probably resulting from dephosphorylation of IP_3 and IP_2 and from slow hydrolysis of phosphatidylinositol by PLC (*2*). In subsequent experiments phosphoinositide (PI) hydrolysis was measured by incubating the cells with carbachol for 30 minutes to allow maximal IP_1 accumulation, while blocking further dephosphorylation by LiCl (*23*) and monitoring IP_1 (*24*). The ED_{50} for carbachol-stimulated PI hydrolysis was $6 \times 10^{-6} M$; a maximal stimulation of 4.5-fold was achieved at $10^{-3} M$ (Fig. 2A). Neither biochemical response could be evoked in nontransfected cells by carbachol (see below).

The muscarinic antagonist atropine has similar affinity for different mAChR subtypes, whereas the antagonist pirenzepine has greater affinity for M1 than M2 mAChRs (*6*). We previously established the cloned porcine atrial mAChR as M2 by its low affinity for pirenzepine and high affinity for atropine (*12*). Each compound was equally effective in antagonizing the effects of carbachol on AC and on PI hydrolysis, confirming that both responses were mediated by the M2 mAChR (Fig. 2B). The difference in the affinity of the two antagonists for the M2 subtype was reflected by their differential potency in blocking the effects of carbachol (Fig. 2B).

In view of the possibility that the 85-fold difference in ED_{50} values between the two responses could have resulted from the different conditions under which they were measured (Fig. 2A), the ability of an endogenous receptor in CHO cells to regulate these responses was tested under similar conditions. Thrombin-evoked PI hydrolysis and AC inhibition with similar efficacy (ED_{50} = 5 to 10 n*M*); thus, the difference in the response to carbachol is not an artifact of the experimental conditions. To further investigate whether the coupling of the M2 mAChR to PI hydrolysis was less efficient than coupling to AC, transfected cell populations expressing different mAChR levels were studied (Fig. 3). The stimulation of PI hydrolysis was highly dependent on receptor number, reaching a plateau above 1.45×10^6 receptors per cell (Fig. 3A). In contrast, the inhibition of AC was similar at each receptor number studied (Fig. 3B). Neither effect could be evoked in nontransfected cells, demonstrating that endogenous mAChRs [less than 100 sites per cell (*12, 13*)] do not contribute to the response seen here.

The PTX-sensitive G proteins include G_i, G_0 (*1*), and a G protein that may couple PLC (G_p) (*1, 3*). In some cell types G_p is not sensitive to PTX, suggesting that there is more than one G_p species (*19, 20*). To determine whether PTX could distinguish between the coupling of a single mAChR subtype to different effectors in the same cell, we investigated its effects on carbachol-stimulated responses in the transfected cells. The AC response was 8.7-fold more sensitive to PTX than the PI response (Fig. 4). These results are surprising because the less efficiently coupled PI response might be expected to have higher PTX sensitivity if both responses were coupled through the same G protein. To investigate the relation between the PTX sensitivity of the biochemical responses and the in vivo ADP-ribosylation of PTX substrates in the transfected cells, we assayed membranes prepared from cells treated with various PTX concentrations to determine the residual amount of unmodified PTX substrate by in vitro ADP-ribosylation (Fig. 4). At concentrations sufficient to abolish the AC but not the PI response (1 to 30 ng/ml), the membranes contained significant levels of nonribosylated PTX substrate, whereas at higher concentrations, sufficient to abolish both responses, nonribosylated PTX substrate was not detected (Fig. 4). Thus, different G proteins may couple the M2 mAChR to these effector systems in CHO cells, or alternatively the two responses are coupled differentially via the same G protein.

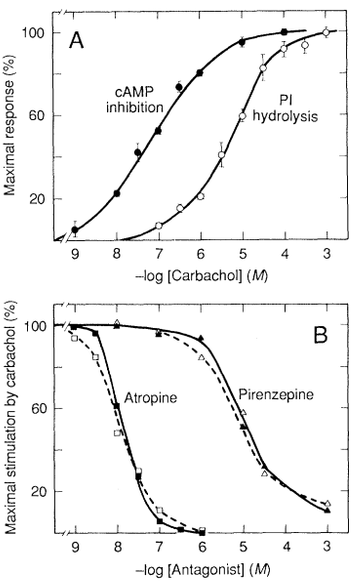

Fig. 2. The effect of carbachol on cAMP formation and PI hydrolysis and its antagonism by atropine and pirenzepine. (**A**) Inhibition of forskolin-induced accumulation of cAMP (●) and stimulation of PI hydrolysis (○) by carbachol (means ± SEM of three and four experiments, respectively). Cells expressing 1.45×10^6 mAChRs per cell were equilibrated with IBMX (100 μ*M*) and treated with forskolin (10 μ*M*) and varying concentrations of carbachol (*25*). The level of cAMP in nontreated cells and in cells treated with IBMX alone was 4.0 ± 0.2 and 7.5 ± 0.5 pmol per 10^6 cells, respectively. Forskolin increased cAMP levels to 124.0 ± 24.0 and 35.1 ± 12.2 pmol per 10^6 cells in the absence or presence of 0.1 m*M* carbachol, respectively (that is, the maximal inhibition was 76.4 ± 15.0%). Cells of the same population were assayed for carbachol-stimulated PI hydrolysis (*24*). Accumulated IP_1 levels were 33.8 ± 5.2 fmol per 10^6 cells in nonstimulated cells and 153.1 ± 32.6 fmol per 10^6 cells in cells stimulated with 1 m*M* carbachol (that is, the maximal stimulation was of 4.5 ± 0.5-fold. (**B**) Effect of the muscarinic antagonists atropine (■ and □) and pirenzepine (▲ and △) on carbachol-induced inhibition of cAMP accumulation (■ and ▲) and stimulation of PI hydrolysis (□ and △), at 0.1 m*M* carbachol. Representative results from one of two experiments are shown. Half-maximal inhibition of both actions of carbachol was achieved at $1.3 \times 10^{-8} M$ atropine or $1 \times 10^{-5} M$ pirenzepine.

Table 1. Computer-fitted parameters of agonist (carbachol or oxotremorine) binding to homogenates of the transfected CHO cells. The data were derived from analysis of the competition displacement data shown in Fig. 1 (*14*). In all cases the best fit was with a two-site model. Values are the means ± SEM of at least three experiments.

Pretreatment with PTX	Treatment with GTPγS	K_H* (μ*M*)	K_L† (μ*M*)	High affinity sites (% of total)
		Carbachol		
−	−	1.57 ± 0.29	160.9 ± 15.7	30.3 ± 1.9
−	+	1.64 ± 1.32	106.3 ± 13.9	8.7 ± 3.1
+	−	1.60 ± 0.33	100.3 ± 11.2	8.7 ± 3.9
		Oxotremorine		
−	−	0.2 ± 0.07	6.0 ± 0.1	24.0 ± 0.3
−	+	0.2 ± 0.02	6.0 ± 0.1	9.9 ± 0.3
+	−	0.2 ± 0.02	10.0 ± 0.4	5.1 ± 0.1

*The dissociation constant for binding to high affinity sites. †The dissociation constant for binding to low affinity sites.

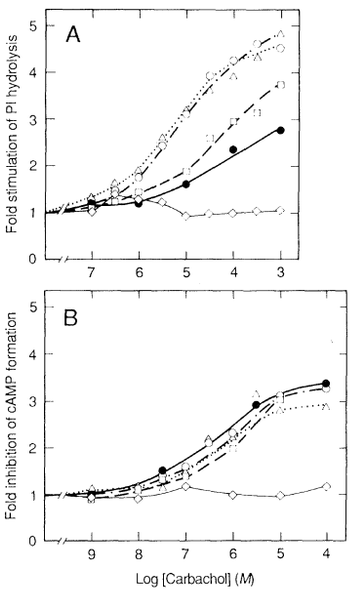

Fig. 3. The effect of mAChR expression level on the biochemical response induced by carbachol. Transfected cell populations expressing various levels of the recombinant mAChR (*13*) were assayed for carbachol-induced activation of PI hydrolysis (**A**) and inhibition of forskolin-induced cAMP formation (**B**), as in Fig. 2. The fold stimulation depicts the ratio of IP_1 levels in the presence of carbachol relative to the level in its absence, and the fold inhibition depicts the ratio of cAMP level in the absence of carbachol relative to the levels in its presence. The values are means of three experiments, where the standard errors were less than 10% of the means. The receptor levels in the different cell populations, as determined by Scatchard analysis of [^{3}H]QNB binding (*15*), were (in QNB sites per cell) 2.4×10^5 (●), 6×10^5 (□), 1.45×10^6 (○), and 2.5×10^6 (△). Nontransfected CHO cells (◇) had less than 100 sites per cell.

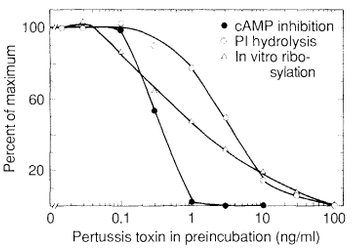

Fig. 4. Differential inhibition of the effects of carbachol by PTX. Carbachol-induced inhibition of AC (●) and activation of PI hydrolysis (○) were measured in cells treated with varying concentrations of PTX (4.5 hours at 37°C). The values are means of four experiments, where the standard errors were normally less than 15% of the means. Cyclic AMP and IP_1 levels determined in cells not stimulated with carbachol were not significantly affected by such treatment with PTX. Half-maximal inhibition was at 0.3 and 2.6 ng of PTX per milliliter for the cAMP and PI responses, respectively. Membranes prepared from parallel incubations of cells with PTX were also subjected to in vitro ADP-ribosylation by PTX and analyzed by SDS gel electrophoresis. The ^{32}P-labeled nicotinamide adenine dinucleoide (NAD) labeled PTX substrates migrated as a band of M_r 40,500 ± 330 (n = 4 experiments), which was excised from the gels and assayed for radioactivity. The results of a representative experiment (△) are expressed as the percentage of the maximal in vitro ADP-ribosylation, occurring in membranes from nontreated cells, where the amount of [^{32}P]NAD incorporated was 240 fmol per milligram of membrane proteins.

Although we have demonstrated that a single M2 mAChR subtype can regulate multiple biochemical events, its inefficiency in mediating PI hydrolysis in CHO cells raises questions about the biological significance of this coupling. The high level of recombinant receptor expression could allow the detection of inefficient coupling to G_p that may not occur at lower receptor levels. Nevertheless, the level of carbachol-induced PI hydrolysis we observed is comparable to that mediated by endogenous thrombin receptors present at physiological levels. The PTX sensitivity of mAChR-stimulated PI turnover in CHO cells contrasts with the lack of sensitivity in other cells (*20*), suggesting the involvement of different G proteins. The low efficiency of M2 mAChR-mediated PI hydrolysis could reflect its weak interaction with the G_p in CHO cells. Alternatively, G_p levels could be low relative to G_i and therefore require higher receptor levels for efficient coupling to PLC.

Previous studies have suggested that different mAChR subtypes mediate different responses. For example, in rat brain, pirenzepine is 15-fold more potent in antagonizing carbachol-induced PI hydrolysis than AC inhibition, suggesting that these responses are mediated by the M1 and M2 subtypes, respectively (*8*). Here we have demonstrated that a single mAChR subtype can be coupled to more than one effector system. These findings indicate that the ability of an individual receptor subtype to recognize various effector systems is differential, rather than exclusive, and may be determined by the cellular context in which it is evoked.

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13. To increase the expression of the mAChR cDNA through coamplification of DHFR sequences, we cultured the transfected cells in the presence of increasing concentrations of methotrexate (up to 500 n*M*) (*12*). Populations expressing from 2.4×10^5 to 2.5×10^6 QNB sites per cell were thus selected. Nontransfected CHO cells, as well as cells transfected with a similar vector containing OKT4 complementary DNA sequences (D. Smith and D. Capon, unpublished results) expressed less than 100 QNB sites per cell.
14. Binding studies were carried out as described (*12*). Nonspecific binding was determined in the presence of 10 μ*M* atropine and was below 15 or 5% of the total binding for intact cells and homogenates, respectively. Saturation curves were analyzed by the LIGAND computer program (*15*). Competition binding experiments, in which the binding of [^{3}H]QNB was displaced by carbachol or oxotremorine, were analyzed by computer-fit least-squares analysis, and the best fit was determined by analysis of variance (*16*).
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24. Polyphosphoinositide hydrolysis was assayed as follows: Confluent monolayer cultures were labeled with [^{3}H]myoinositol (2 μCi/ml) for 40 hours. Monolayers were then washed and incubated (37°C) for 45 minutes in phosphate buffered saline (PBS), followed by a 15-minute incubation in PBS containing 10 m*M* LiCl. The cells were suspended (1.5×10^6 cells per milliliter) in PBS containing LiCl and assayed in 1-ml aliquots. Because preliminary experiments revealed that carbachol stimulates a rapid increase in IP_3 and IP_2 levels followed by a slower and linear accumulation of IP_1 (see text), an

incubation time of 30 minutes was chosen to allow for dephosphorylation of IP_3 and IP_2 to form IP_1, while preventing further dephosphorylation of the latter by including LiCl (*24*). Under these conditions IP_1 accumulation served as indicator of PLC-catalyzed hydrolysis of phosphoinositides. After incubation with agonists or antagonists or both, the reaction was stopped by precipitation with trichloroacetic acid. The supernatants were extracted with diethyl ether, neutralized, and applied to Dowex-100 columns, on which inositol phosphates were separated (*24*).

25. The inhibition of AC-catalyzed formation of cAMP was assayed as follows: Confluent cells were harvested as in (*18*), washed once, and resuspended in PBS (5×10^5 cells per milliliter). Aliquots (1 ml) were equilibrated for 20 minutes at 37°C with 100 μM IBMX. Muscarinic agonists, antagonists, and forskolin (10 μM) were then added, and incubation was continued for 10 minutes. The cells were pelleted, resuspended in buffer, boiled for 10 minutes, and centrifuged (5 minutes, 10,000*g*), and the supernatants were assayed for cAMP content by radioimmunoassay (*22*).

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Physiological Role of Silent Receptors of Atrial Natriuretic Factor

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A ring-deleted analog of atrial natriuretic factor—des[Gln^{18}, Ser^{19}, Gly^{20}, Leu^{21}, Gly^{22}] ANF_{4-23}-NH_2 (C-ANF_{4-23})—binds with high affinity to approximately 99% of ANF receptors in the isolated perfused rat kidney. In this preparation, C-ANF_{4-23} is devoid of detectable renal effects and does not antagonize any of the known renal hemodynamic and natriuretic actions of biologically active ANF_{1-28}. In contrast, both C-ANF_{4-23} and ANF_{1-28} increase sodium excretion and decrease blood pressure in intact anesthetized rats. This apparent contradiction is resolved by the finding that the ring-deleted analog markedly increases plasma levels of endogenous immunoreactive ANF in the rat. The results show that the majority of the renal receptors of ANF are biologically silent. This new class of receptors may serve as specific peripheral storage-clearance binding sites, acting as a hormonal buffer system to modulate plasma levels of ANF.

ADMINISTERED ATRIAL NATRIURETIC factor (ANF) has important effects on such body functions as the regulation of renal function, salt balance, plasma volume, and blood pressure (*1*). Many, if not all, of these actions, are assumed to be mediated by guanosine 3′,5′-monophosphate (cGMP), which is generated as ANF interacts with its biological receptors (B-ANF receptors) (*2*). Specific high-affinity binding sites of ANF have been described in several tissues; in kidney they are localized mainly in vascular and glomerular structures of the cortex (*1–3*). Recent studies in the isolated perfused rat kidney show a remarkable identity between the specific binding curve of ANF in the kidney cortex and the dose-response curves of such renal effects of the hormone as increase in glomerular filtration rate (GFR), increase in urinary excretion of fluid and electrolytes, and vasorelaxation of preconstricted renal vasculature (*4*). There are, however, major discrepancies between the kinetics of ANF-specific binding and the dose-response curve of its effect on cGMP accumulation (*2, 5*). Furthermore, in vascular smooth muscle and endothelial cells in culture, some analogs of ANF that effectively compete for specific binding sites are very weak agonists and do not antagonize the ANF-induced increase in cGMP (*6*). These data raise the possibility that either cGMP is not the sole mediator of ANF actions or that there is a separate class of biologically silent ANF receptors (C-ANF receptors).

In this report we address the question of whether there is a significant number of C-ANF receptors in the kidney and explore the issue of the physiological role of these receptors. For these purposes we used a combined in vitro–in vivo approach, which relates specific binding curves to the functional effects of biologically active ANF_{1-28} (*7*) and of the ring-deleted analog C-ANF_{4-23} (des[Gln^{18}, Ser^{19}, Gly^{20}, Leu^{21}, Gly^{22}] ANF_{4-23}-NH_2) (*8*). These peptides were chosen because ANF_{1-28} is the natural form of biologically active ANF (*7*), whereas C-ANF_{4-23} competes effectively with biologically active atrial peptides for binding sites but is devoid of agonist or antagonist action on the generation of cGMP in vascular smooth muscle and endothelial cells in culture (*9*).

C-ANF_{4-23} effectively competed for the overwhelming majority of specific binding sites of ^{125}I-labeled ANF_{1-28} in isolated perfused rat kidney (Table 1 and Fig. 1). This analog ($10^{-7} M$) almost completely inhibited the specific binding of ^{125}I-labeled ANF_{1-28} to whole-kidney tissue, cortex, and inner stripe of the outer medulla. From these data it can be estimated that C-ANF_{4-23} can occupy close to 99% of the binding sites of ANF in these kidney regions (Table 1). In the renal papilla, which contains less than 2% of the total binding sites of ANF_{1-28} in the kidney (*4*), C-ANF_{4-23} bound to a lesser extent than ANF_{1-28} but still was able to occupy approximately 60% of the specific binding sites of ^{125}I-labeled ANF_{1-28} (Table 1, Fig. 1C). The apparent affinities of C-ANF_{4-23} (expressed as S_{50}, the perfusate concentration of the peptide required to decrease the specific binding of ^{125}I-labeled ANF_{1-28} to half the maximal level) for binding sites in whole-kidney tissue and kidney cortex were very high, albeit about eightfold lower than the corresponding apparent affinities of ANF_{1-28} (Fig. 1, A and B).

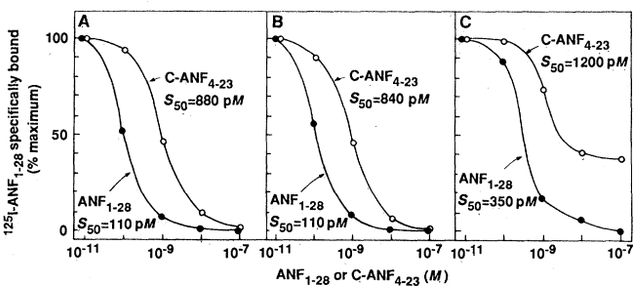

Fig. 1. Competition for binding between ^{125}I-labeled ANF_{1-28} and ANF_{1-28} or the ring-deleted analog C-ANF_{4-23} in whole-kidney tissue (**A**), cortex (**B**), and papilla (**C**) in isolated rat kidney. Kidneys were perfused in a closed-circuit system in the nonfiltering mode as described (*16*). ^{125}I-labeled ANF_{1-28} (4 pM) and the concentrations of ANF_{1-28} or C-ANF_{4-23} indicated on the abscissas were added together to the perfusate at the beginning of the perfusion. After 50 minutes, perfusion was terminated and specific binding of ^{125}I-labeled ANF_{1-28} to whole-kidney tissue, cortex, and papilla was determined as described (Table 1) (*4*). Results (average of two kidneys per concentration point) are expressed as percentage of maximal specific binding (ordinate) against perfusate concentration of peptides (abscissa). S_{50}, perfusate concentration of peptide required to decrease specific binding of ^{125}I-labeled ANF_{1-28} to half the maximal level.

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